PARASITOLOGY

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VOLUME 48, 1958

CAMBRIDGE
AT THE UNIVERSITY PRESS
1958

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)
and published by the Cambridge University Press
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.
Agent in Canada: The Macmillan Company of Canada Limited

CONTENTS

Nos. 1, 2 (May 1958)

PAGE

MEYER, H., OLIVEIRA MUSACCHIO, M. DE and ANDRADE MENDONÇA, I. DE. Electron microscopic study of *Trypanosoma cruzi* in thin sections of infected tissue cultures and of blood-agar forms. (With Plates I–VI) . . . 1

INGLIS, WILLIAM G. A revision of the nematode genus *Meteterakis* Karve, 1930. (With 22 Figures in the Text) 9

BIRD, ALAN F. Further observations on the structure of nematode cuticle. (With Plates VII and VIII) 32

ARTHUR, D. R. New species of *Ixodes* ticks from eastern Africa, with a description of the male and nymph of *Ixodes oldi* Nuttall, 1913. (With 91 Figures in the Text) 38

CLARKSON, M. J. Life history and pathogenicity of *Eimeria adenoeides* Moore & Brown, 1951, in the turkey poult. (With 6 Figures in the Text) . . 70

JACK, K. M. Notes on three species of *Pterygosoma* (Acarina: Pterygosomidae) from Gerrhosaurid lizards. (With 3 Figures in the Text) 89

JAIN, G. P. On the egg and miracidium of *Paryphostomum mehrai* (Faruqui). (With 3 Figures in the Text) 96

JOYNER, L. P. Experimental *Eimeria mitis* infections in chickens. (With 6 Figures in the Text) 101

WALTERS, V. ANN. Structure, hatching and size variation of the spores in a species of *Nosema* (Microsporidia) found in *Hyalophora cecropia* (Lepidoptera). (With 1 Figure in the Text) 113

LEE, D. L. A note on a species of *Syngamus* found in the intestine of *Turdus pilaris*. (With 5 Figures in the Text) 121

ARTHUR, D. R. A review of some ticks (Acarina: Ixodidae) of sea birds, including a consideration of other cognate material. I. (With 13 Figures in the Text) . 124

BELL, E. J. and SMYTH, J. D. Cytological and histochemical criteria for evaluating development of trematodes and pseudophyllidean cestodes *in vivo* and *in vitro*. (With 2 Figures in the Text) 131

THURSTON, JUNE P. The oxygen uptake of *Trypanosoma lewisi* and *Trypanosoma equiperdum*, with especial reference to oxygen consumption in the presence of amino-acids. (With 7 Figures in the Text) 149

THURSTON, JUNE P. The effect of some metabolic inhibitors on the oxygen uptake of *Trypanosoma lewisi* and *Trypanosoma equiperdum*. (With 14 Figures in the Text) 165

SPRENT, J. F. A. Observations on the development of *Toxocara canis* (Werner, 1782) in the dog. (With Plate IX and 19 Figures in the Text) . . . 184

BISHOP, ANN. An analysis of the development of resistance to metachloridine in clones of *Plasmodium gallinaceum* 210

Nos. 3, 4 (November 1958)

PAGE

Crofton, H. D. Nematode parasite populations in sheep on lowland farms. IV. The effects of anthelmintic treatment. (With 7 Figures in the Text) . 235

Crofton, H. D. Nematode parasite populations in sheep on lowland farms. V. Further observations on the post-parturient rise and a discussion of its significance. (With 6 Figures in the Text) 243

Crofton, H. D. Nematode parasite populations in sheep on lowland farms. VI. Sheep behaviour and nematode infections. (With 2 Figures in the Text) 251

Foster, R. The effects of trematode metacercariae (Brachylaemidae) on the slugs *Milax sowerbii* Férussac and *Agriolimax reticulatus* Müller. (With Plate X) 261

Paterson, Nellie F. External features and life cycle of *Cucumaricola notabilis* nov. gen. et sp., a copepod parasite of the holothurian, *Cucumaria*. (With 11 Figures in the Text) 269

Soliman, K. N. *Globidium gilruthi* (Chatton, 1910) infection in the digestive tract of sheep and goats in Egypt 291

Gresson, R. A. R. The gametogenesis of the digenetic trematode *Sphaerostoma bramae* (Müller) Lühe. (With Plates XI and XII) 293

Foster, R. Infestation of the slugs *Milax sowerbii* Férussac and *Agriolimax reticulatus* Müller by trematode metacercariae (Brachylaemidae). (With 2 Figures in the Text) 303

Erasmus, David A. Studies on the morphology, biology and development of a strigeid cercaria (*Cercaria X* Baylis 1930). (With Plate XIII and 6 Figures in the Text) 312

Thomas, J. D. Studies on *Crepidostomum metoecus* (Braun) and *C. farionis* (Müller), parasitic in *Salmo trutta* L. and *S. salar* L. in Britain. (With 10 Figures in the Text) 336

Tetley, J. H. The sheep ked, *Melophagus ovinus* L. I. Dissemination potential 353

Tetley, J. H. The sheep ked, *Melophagus ovinus* L. II. Keds acquired by a lamb from its mother. (With 5 Figures in the Text) 364

Dasgupta, Biswapati. A new schizogregarine, *Mattesia orchopiae* n.sp., in a flea of squirrels in England. (With 14 Figures in the Text) 375

Rothschild, Miriam. The bird fleas of Fair Isle. (With 10 Figures in the Text) 382

Jain, G. P. On *Cercaria mehrai* Faruqui, 1930 with notes on its life-history. (With 4 Figures in the Text) 413

Dasgupta, B. and Meedeniya, Kamani. The vector of *Hepatozoon sciuri*. (With 7 Figures in the Text) 419

McConnachie, Elspeth W. Factors affecting the growth and behaviour of axenic strains of *Entamoeba invadens* Rodhain, 1934 423

Lee, D. L. On the morphology of the male, female and fourth-stage larva (female) of *Hammerschmidtiella diesingi* (Hammerschmidt), a nematode parasitic in cockroaches. (With 8 Figures in the Text) 433

PAGE

LEE, D. L. Digestion in *Leidynema appendiculata* (Leidy, 1850), a nematode parasitic in cockroaches. (With 4 Figures in the Text) 437

TATCHELL, R. J. The physiology of digestion in the larvae of the horse bot-fly, *Gasterophilus intestinalis* (de Geer). (With 8 Figures in the Text) . . 448

SMITH, KENNETH M. A study of the early stages of infection with the tipula iridescent virus. (With Plates XIV–XVII) 459

THURSTON, JUNE P. The effect of immune sera on the respiration of *Trypanosoma brucei in vitro*. (With 2 Figures in the Text) 463

REES, GWENDOLEN. A comparison of the structure of the scolex of *Bothriocephalus scorpii* (Müller 1776) and *Clestobothrium crassiceps* (Rud. 1819) and the mode of attachment of the scolex to the intestines of the host. (With Plates XVIII and XIX and 31 Figures in the Text) 468

BAILEY, L. The epidemiology of the infestation of the honeybee, *Apis mellifera* L., by the mite *Acarapis woodi* Rennie and the mortality of infested bees. (With 5 Figures in the Text) 493

INDEX OF AUTHORS 507

INDEX OF SUBJECTS 509

ELECTRON MICROSCOPIC STUDY OF *TRYPANOSOMA CRUZI* IN THIN SECTIONS OF INFECTED TISSUE CULTURES AND OF BLOOD-AGAR FORMS

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(With Plates I–VI)

The ultrastructure of *Trypanosoma cruzi* has been described in an earlier paper, Meyer & Porter (1954). In the unsectioned forms of this parasite, as well as in the few forms from tissue cultures which had been examined at that time, a fine striation was observed in the cytoplasm of the trypanosome. However, the main part of the body was too thick for the penetration of the electron beam, and nothing could be seen of its inner structure. The blepharoplast in those preparations is represented by a vacuole-like formation which is crossed by some dense fibres. The whole body is covered by a fine sheath. The flagellum consists of an axial fibre bundle, although the number of its fibres could not be established with certainty. It is covered by a fine sheath which is clearly separated from the one which covers the body part of the trypanosome, showing that the flagellum is fixed to the body surface by adhesion only.

The investigations have been continued since, and some of the results obtained recently will be discussed in this paper.

MATERIAL AND METHODS

The parasite was obtained in tissue cultures and studied in all the forms in which it appears in these cultures inside and outside the tissue cells (Meyer, 1951). Some unsectioned preparations have been made by transferring the cells, following the technique of Porter, Claude & Fullam (1945), to the electron microscope grids. These preparations have been compared with thin sections of the same material, and also thin sections of the blood-agar forms which had been used for the contamination of the tissue cultures were examined. The trypanosome strain 'Bustamante', used for this investigation, had been isolated from human blood in the State of Goiás.

When the tissue cultures showed a rich contamination they were prepared for transfer, or the whole culture was embedded and cut in thin sections. For transfer the thick part of the culture was removed, and the remaining cells and parasites were fixed in osmic acid vapours for 10–20 min. For embedding the whole cultures were immersed for 30 min. in 1% osmic acid, buffered with veronal acetate to pH 7·6–7·8. They were fixed on the cover-slips and kept there for washing and dehydration up to 90% methyl alcohol. The interesting areas previously marked were cut out with a sharp cataract knife, and the process of dehydration and

embedding in *n*-butyl methacrylate was continued, according to the technique elaborated by Palade (1952*a*) for small tissue fragments. The blood-agar forms were carefully washed 3 times in Tyrode solution, in order to remove the protein of the nutrient medium, and fixed then by adding 2 drops of 1 % osmic acid to *ca.* 2 ml. of suspension, during 10–20 min. After several washings in distilled water some of the fixed trypanosomes were dropped on parlodion-coated grids for direct observation in the electron microscope without sectioning. The remaining parasites were dehydrated and embedded in *n*-butyl methacrylate. The embedded material was sectioned with the Porter-Blum microtome and all preparations were examined in the Philips electron microscope with 80 and 100 kV.

OBSERVATIONS

As it is seen in Pl. I, fig. 2, it has been possible with this technique to cut well-spread cells of the growth area which contain the parasite in leishmania form. We found it considerably more difficult to get satisfactory sections through the crithidia and the adult form of the trypanosome. In the later phases of the cycle the host cell is completely filled with parasites and they are superimposed on one another. Since *in vivo* they are moving continuously in all directions, even inside the cells, it has not yet been possible to get a whole trypanosome in one section. Only parts of them are always in the cutting plane (Pl. IV, figs. 18–20). We had the same difficulty with the free, extracellular forms from tissue cultures and with the trypanosomes from the blood-agar medium.

Pl. I, fig. 2, shows that every leishmania body has a different position in the cell, because this form also is in constant movement, a fact which can be observed readily *in vivo* (Meyer & Xavier de Oliveira, 1948). The body of the parasite is covered by a double membrane and contains a large nucleus, some vacuoles and lipid granules, a canal system of about 2000 Å. width and a finer one of 500 Å. In most of these forms, depending on the cutting plane, we see a dark, slightly bent inclusion, which is situated near the nucleus, the kinetonucleus. Near this formation we see a short fibre bundle, the flagellum, which has its origin in several dark bodies, the basal corpuscles. With the light microscope no separation of these structures is possible. In fixed and stained preparations we see in this region a dark stained line and call it the blepharoplast or kinetonucleus (Pl. I, fig. 1). The ground substance shows in the electron microscope a dense granulation with no visible striation.

With higher magnification we see that the kinetonucleus is represented by a vacuole-like space which contains an inclusion of high electron density. In longitudinal sections this inclusion shows a lamellar, almost spiral-like structure (Pl. I, figs. 2*a*, 3, 4; Pl. II, fig. 8; Pl. III, figs. 12, 14). In cross-sections it covers a circular ring-like area (Pl. I, fig. 4; Pl. II, figs. 5, 6). It is not separated from the surrounding space by a limiting membrane. The centre is lighter and more homogeneous than the periphery, and is composed of small irregular areas with fine granules of about 200 Å in the centre. The structure of the dark masses is irregular too; it seems to be composed of many spots, approximately 400 × 1200 Å. in size, and each spot has a central point which is still darker than the surrounding areas. In some

sections a certain radial arrangement of the masses can be observed (Pl. II, fig. 6). The lighter central masses go over directly into the darker peripheral ones.

Depending on the direction of the cut, it can be seen that the vacuole-like space continues directly into the large canal system. In Pl. I, fig. 3, this shows septa or lamellae similar to those which have been described by Palade (1952*b*) and others, as typical for mitochondria in tissue cells.

The basal corpuscles which give origin to the axial fibre bundle of the flagellum are situated near the kinetonucleus, though separated from it (Pl. I, fig. 2; Pl. II, figs. 7, 8; Pl. III, figs 12, 14). They continue directly into the axial thread. They are darker than the surrounding cytoplasm and the axial fibres and seem to be thickenings of these (Pl. II, fig. 7). In many preparations they show a dense, longitudinal striation in parallel arrangement, and even a transversal banding can be observed occasionally in the striae (Pl. II, fig. 8). It has not been possible to count the exact number of the basal corpuscles, but the number of the axial fibres can be counted in cross-sections of the flagellum (Pl. I, fig. 4; Pl. II, figs. 9, 10). There is a peripheral number of 9 and a central pair of two fibres. The number and arrangement of these fibres is the same as that seen in cilia and other flagella by various investigators (Fawcett & Porter, 1954; Anderson, Saxe & Beams, 1956). The diameter of the single fibres is about 300 Å.

The axial fibres of the flagellum run parallel and show in most of the preparations no transverse structures. In some sections, however, at the base of the flagellum a regular periodicity has been observed (Pl. II, figs. 7, 8). This is too regular to be considered an artifact. One period measures about 500 Å. The matrix in which the axial fibres are embedded is transparent and shows no structure at all.

It is interesting to notice that the surface membrane of the parasite is pushed inwards by the basal corpuscles; it then makes a turn and covers the whole bundle of the axial fibres (Pl. II, figs. 7, 8; Pl. IV, fig. 18). During the development, e.g. during the migration of the kinetonucleus to the posterior tip of the trypanosome, an invagination is formed, by which the flagellum comes to lie eventually at the surface of the body, separated from it by its own sheath, as we (Meyer & Porter, 1954) saw in the unsectioned parasites and as is seen in cross-sections of the trypanosome (Pl. II, fig. 10; Pl. VI).

In cross-sections of both the leishmania and trypanosome forms of the parasite, it is seen that the fine striation which had been observed in the unsectioned parasites is only a surface structure, the periplast. It is situated below the thin sheath which covers the whole organism. The size of the single fibres is about 450 Å. wide and 450 Å. deep (Pl. I, fig. 4; Pl. II, figs. 5, 10).

The nucleus of the parasite has a dense granular structure and shows lighter and darker areas. The granules have a diameter of 50–100 Å. approximately. No mitotic figures have been found. The many images which have been examined of the dividing forms seem to indicate that the division of the parasite begins in the basal corpuscles. In some preparations, these, together with their axial fibres, are already divided into two portions (Pl. III, fig. 12), when the kinetonucleus is still an uninterrupted line. Only later does the kinetonucleus bend down in the middle, when the nucleus often still shows no signs of division. A more homogenous

structure, resulting from the disappearance of the darker areas, seems to precede its final fission (Pl. I, fig. 2*a*; Pl. III, fig. 13). The division of the cytoplasm is still more delayed, many forms can be found with two nuclei, two kinetonuclei and two flagella, with the cytoplasm still undivided (Pl. III, fig. 15).

In the more advanced forms of the cycle, when the body has become more elongated, we find in the cytoplasm of the trypanosome a group of short striae, regularly spaced, 300 Å. apart and about 250 Å. wide. They are situated in the region between the nucleus and the kinetonucleus (Pl. IV, fig. 20). This latter, together with the basal bodies of the flagellum, comes to lie in the posterior half of the body. The basal corpuscles, following the migration of the kinetonucleus —or pushing the kinetonucleus backwards—are forming the invagination of the surface membrane described above, and this brings about the separation of the flagellum, covered by its own sheath and increased considerably in length, from the main body.

The small space which contains the dark masses of the kinetonucleus gradually becomes larger and forms a great vacuole at the posterior tip of the parasite. In the light microscope, in fixed and stained preparations, a spherical formation is seen in this phase of the cycle, just below the pointed tip of the trypanosome. It is stained uniformly dark with chromatin dyes and is commonly called the blepharoplast (Pl. IV, fig. 19). The many images of this region which we have examined with the electron microscope, in unsectioned preparations as well as in thin sections, do not leave any doubt that we are dealing here with a vacuole whose content is transparent (Pl. III, figs. 16, 17; Pl. V, figs. 20*a*–22).

Owing to the great difficulties which we had in obtaining the entire trypanosome in one section we cannot say what becomes of the dark masses of the kinetonucleus in this phase of the development. In all the adult forms which have been examined so far we did not identify them with certainty. In some sections it seems as if they were losing their structure in the fluid of the great vacuole at the end of the body (Pl. II, fig. 11). For the same reason we cannot establish the exact position of the basal corpuscles in this phase. In some of the cross-sections they can be seen near the wall of the great vacuole.

In the cytoplasm of the adult trypanosome, which is now more homogeneous, we see, besides the nucleus, the small canals and the group of short striae, some dark granules, probably of a lipid nature. No mitochondria have been identified with certainty.

The unsectioned trypanosomes from tissue cultures show that the body of the parasite, beyond the vacuole, ends in an extension of varying length. It is in contact with the proximal part of the body by a system of fibres which run over the vacuole and which are often dislodged by the large size of this (Pl. III, figs. 16, 17; Pl. V, figs. 20*a*–22).

DISCUSSION

There are many problems which are related to *Trypanosoma cruzi*. They do not concern the parasite alone, but also the host cell which determines its life cycle, e.g. the duration of the cycle and the number of trypanosomes at the end of it

(Meyer & Xavier de Oliveira, 1948). In this paper our attention has been focused on the parasite only. The changes which are produced in the parasitized cells will be discussed in another paper.

The images which have been so far obtained in the electron microscope show very clearly the distinction between the two structures—kinetonucleus and blepharoplast—in the leishmania and crithidia forms, which could not be obtained by light microscopy, or with the electron microscope in unsectioned preparations. The blepharoplast is the row of basal corpuscles which give origin to the axial fibres of the flagellum just as similar structures give origin to the cilia of any ciliated cell. The blepharoplast has, apparently, no connexion with the kinetonucleus, and we do not know whether the migration of the two structures during the cycle is entirely passive or which of the two plays the active part in it.

We see further that the kinetonucleus is surrounded by a vacuole-like space. This is in direct continuation with the large canal system, which has been observed in the cytoplasm, and which shows in some preparations the lamellar structure that is typical for mitochondria. The chemical nature of the dark masses which compose the kinetonucleus has to be studied by other methods. Positive Feulgen reactions have been reported of this region in other trypanosomes by Cosgrove & Andersen (1954). Couceiro & Freitas (1950) obtained positive reactions for polysaccharides in the blepharoplast of the metacyclic forms of *T. cruzi* grown in an artificial medium.

The kinetonucleus has certainly an important role in the multiplication of the parasite. The many images of dividing forms which have been obtained and examined during these investigations suggest that an increase of its dark masses from the centre to the periphery takes place and that this brings about division of the whole unit. No structures have been found which suggest that the leishmania and crithidia forms undergo any form of division other than binary fission. If the division of the parasite begins really in the basal bodies of the flagellum, as some of the obtained imagines lead us to believe, this would give an additional importance to these structures and would suggest that they are more than merely the point of origin of the flagellum. Basal corpuscles are often considered to be centrioles (Wenyon, 1926).

Because we could not obtain whole sections of the adult form of the trypanosome we do not yet fully understand the changes which occur in its fine structure during the transformation of the leishmania or crithidia into the adult form. All we can say so far is that the cytoplasm of the adult form of the trypanosome has become more homogenous. The large canal system of the leishmania and crithidia forms has disappeared, and a group of striae has appeared instead, which had not been observed in the leishmania forms, between the nucleus and the kinetonucleus, in the region where in a tissue cell the Golgi apparatus would be located. The blepharoplast, which up to now in the adult trypanosome has been described and considered as a solid chromatin structure, shows in the electron micrographs that it is a great vacuole, which is filled with a transparent, probably liquid content. Our constant failure to identify the typical structure of the kinetonucleus in these forms and the few images obtained of this region like the one shown in Pl. II, fig. 11,

make it probable that the dark masses are dislodged or even dissolved in the liquid of the vacuole. This could explain the fact that the adult trypanosome has lost its capacity for division.

The images which have been obtained of this region from the unsectioned trypanosomes, which have the long extension beyond the vacuole which forms the tip of the parasite, and the fibre system connecting this extension with the body of the trypanosome, suggest strongly that it is an apparatus for penetration. We know that the trypanosome penetrates the cell with this part of its body. We do not yet know what happens after the penetration of a trypanosome into a cell, nor what changes in the fine structure occur during the transformation of the adult into the leishmania form, nor what becomes of the long flagellum of the trypanosome during this transformation. A detailed description of these changes depends on further observations of living cultures, as well as on fixation and sectioning of very restricted areas which technical difficulties have so far prevented.

SUMMARY

Thin sections of *Trypanosoma cruzi* in tissue cultures and from blood agar medium have been examined with the electron microscope.

In the leishmania and crithidia forms of the parasite a neat separation of kinetonucleus and blepharoplast has been obtained and these structures are described. The blepharoplast is the row of basal corpuscles which give origin to the axial fibres of the flagellum.

Division of the parasite seems to begin in the basal corpuscles; it is followed by binary division of the kinetonucleus and only later by fission of nucleus and cytoplasm.

During the development of the leishmania into the crithidia form, the kinetonucleus and basal bodies are dislocated towards the posterior half of the trypanosome. The migration of these two structures and the consequent lengthening of the flagellum causes the surface membrane of the flagellum to be pushed inwards to form a deep invagination, so that the flagellum comes to the surface of the body and is separated from it along its whole length by its own sheath.

In the adult form of the parasite a great vacuole is formed near the posterior tip of the trypanosome. It has no definite structure in the electron microscope, is transparent and seems to be filled with a liquid content. The basal corpuscles are situated in this region near the wall of the vacuole. The typical structure of the kinetonucleus could not be identified with certainty in these forms; it seems to be altered by the formation of the vacuole.

The vacuole is crossed by a fibre system which comes from the body of the trypanosome and is often dislodged by the size of the vacuole. This fibre system ends in a sharply pointed process of varying length.

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REFERENCES

ANDERSON, E., SAXE, L. H. & BEAMS, H. W. (1956). *J. Parasitol.* **42**, 11–16.

COSGROVE, W. B. & ANDERSON, E. (1954). The kinetoplast of *Crithidia fasciculata*. *Anat. Rec.* **120**, 813–14.

COUCEIRO, A. & FREITAS, G. (1950). *Commun. 5th Int. Congr. Microbiology.*

FAWCETT, D. W. & PORTER, K. R. (1954). A study of the fine structure of ciliated epithelia. *J. Morph.* **94**, 221–81.

MEYER, H. (1951). *Methods of Medical Research*, **4**, 281.

MEYER, H. & PORTER, K. R. (1954). A study of *Trypanosoma cruzi* with the electron microscope. *Parasitology*, **44**, 16–23.

MEYER, H. & XAVIER DE OLIVEIRA, M. (1948). Cultivation of *Trypanosoma cruzi* in tissue cultures: a four-year study. *Parasitology*, **39**, 91–4.

PALADE, G. E. (1952*a*). A study of fixation for electron microscopy. *J. Exp. Med.* **95**, 285.

PALADE, G. E. (1952*b*). The fine structure of mitochondria. *Anat. Rec.* **114**, 427–52.

PORTER, K. R., CLAUDE, A. & FULLAM, E. (1945). A study of tissue culture cells by electron microscopy. *J. Exp. Med.* **81**, 233–46.

WENYON, C. M. (1926). *Protozoology*, p. 119. London: Baillière, Tindall and Cox.

EXPLANATION OF PLATES I–VI

PLATE I

Fig. 1. Microphotograph of *Trypanosoma cruzi* in tissue culture. Fibroblast with parasites in leishmania form. Bouin-Haematoxylin.

Fig. 2. *T. cruzi* in tissue culture. Thin section of cell in growth area with parasites in leishmania form. Every leishmania form has different position in cell. *N*, nucleus of leishmania body; *K*, kinetonucleus; *F*, fibre bundle of flagellum; *D*, dividing form; *B*, basal bodies of flagellum. × 4000.

Fig. 2*a*. Higher magnification of one of the dividing forms in fig. 2. *N*, nucleus, apparently in beginning of division, structure more uniform than in other forms; *FCr*, cross-section of one of the two flagella. × 20,000.

Fig. 3. *T. cruzi* in tissue culture. Thin section of two leishmania forms in cell. *N*, nucleus; *K*, kinetonucleus in vacuole-like space which continues in mitochondria-like canal system; *B*, part of basal corpuscles of flagellum. × 15,000.

Fig. 4. *T. cruzi* in tissue culture. Thin section of three leishmania bodies in cell, cut in different positions. *N*, nucleus; *K*, kinetonucleus; *KCr*, cross-section of kinetonucleus; *FCr*, cross-section of flagellum, *PCr*, periplast in cross-section. × 15,000.

PLATE II

Fig. 5. *T. cruzi* from blood-agar culture in thin section. *N*, nucleus; K, kinetonucleus in slightly oblique section, showing small irregular areas with dark point in centre. *PCr*, periplast in cross-section. × 26,000.

Fig. 6. *T. cruzi* from blood-agar culture in thin section. *N*, nucleus; *KCr*, kinetonucleus in cross-section. Centre composed of small irregular areas with dark point in the middle, which go over directly into the darker masses of the periphery. No limiting membrane against the surrounding vacuole. Continuation of vacuole into the canal system of the body. × 16,000.

Fig. 7. *T. cruzi* in tissue culture. Thin section of crithidia form in cell. *K*, kinetonucleus; *B*, basal bodies of flagellum; *A*, axial fibres of flagellum with periodic structure; *N*, nucleus × 21,000.

Fig. 8. *T. cruzi* from blood-agar culture. Crithidia form in thin section. *K*, kinetonucleus; *B*, basal corpuscles, show fine striation with transversal banding; *A*, axial fibres of flagellum with periodic structure; *I*, infolding of surface membrane. × 26,000.

Fig. 9. *T. cruzi* in tissue culture. Thin section of intracellular crithidia form. *FCr*, cross-section of flagellum shows nine peripheral and two central fibres. × 17,000.

Fig. 10. *T. cruzi* from blood-agar culture in thin section. Cross-section of distal part of two trypanosomes. *FCr*, cross-section of two flagella, separated from body. *PCr*, body part of trypanosome with periplast in cross-section. × 9,000.

Fig. 11. *T. cruzi* from blood-agar culture in thin section. Cross-section of posterior tip of metacyclic form. *V*, vacuole of kinetonucleus; *K*, kinetonucleus, apparently in disintegration; *BCr*, basal corpuscles of flagellum in cross-section. × 19,000.

PLATE III

Fig. 12. *T. cruzi* in tissue culture. Thin section through dividing intracellular form. *B*, basal corpuscles already divided in two portions; *K*, kinetonucleus still undivided. × 25,000.
Fig. 13. *T. cruzi* in tissue culture. Thin section of dividing leishmania form. *N*, nucleus in division. × 12,000.
Fig. 14. *T. cruzi* from blood-agar culture. Thin, very oblique section of dividing crithidia form. *B*, basal corpuscles; *A*, axial fibres of the flagellum; *K*, kinetonucleus; *P*, periplast. × 25,000.
Fig. 15. *T. cruzi* in tissue culture. Thin section of dividing leishmania form. *N*, nucleus; *K*, kinetonucleus; *B*, fragment of basal corpuscle, flagella not in cutting plane. Cytoplasm still undivided. × 11,000.
Fig. 16. *T. cruzi* from tissue culture. Posterior end of unsectioned extracellular adult form. Fibre system coming from body part, running over vacuole and forming long, pointed tip. *P*, periplast. × 9,000.
Fig. 17. Same as fig. 16.

PLATE IV

Fig. 18. Part of tissue culture cell in thin section, filled with parasites in early crithidia form. Parasites superimposed upon one another, only parts of them in the cutting plane. *N*, nucleus; *K*, kinetonucleus in vacuole-like space, *F*, flagellum; *BCr*, cross-section of basal body; *I*, infolding of surface membrane. × 13,000.
Fig. 19. Microphotograph of tissue culture cell with *T. cruzi* in final phase of cycle. Bouin-Haematoxylin.
Fig. 20. *T. cruzi* in final phase of cycle, thin section of destroyed tissue culture cell, no entire trypanosome in section. *V*, great vacuole at posterior tip of body; *VCr*, vacuole in cross-section; *F*, flagellum; *N*, nucleus; *Str*, group of striae in posterior half of body; *FS*, fibre system over vacuole at posterior tip. × 9,000.

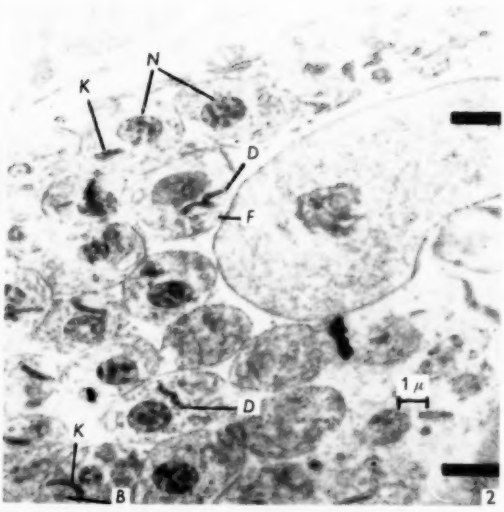
PLATE V

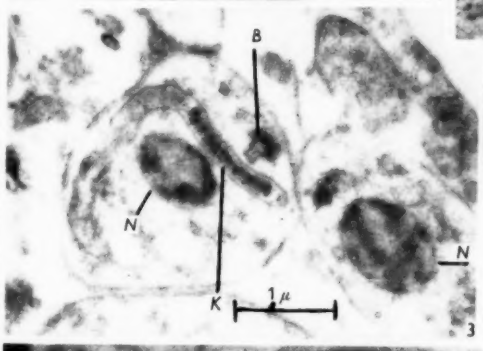
Fig. 20*a*. Higher magnification of posterior tip of trypanosome in fig. 20. Vacuole shows no definite structure. *F*, flagellum; *Str*, striae; *P*, periplast in longitudinal section; *G*, granules of uncertain nature. × 30,000.
Fig. 20*b*. Higher magnification of trypanosome fragment in fig. 20. *N*, nucleus; *Str*, group of striae; *C*, small canals; *VCr*, cross-section of vacuole at posterior tip of another trypanosome, shows no definite structure. × 15,000.
Fig. 21. *T. cruzi* from tissue culture, unsectioned extracellular form. Vacuole formation at posterior tip with transparent content. × 12,000.
Fig. 22. Same as in fig. 21. Fibre system running over vacuole formation at posterior tip of trypanosome, ending in long, pointed process. × 12,000.

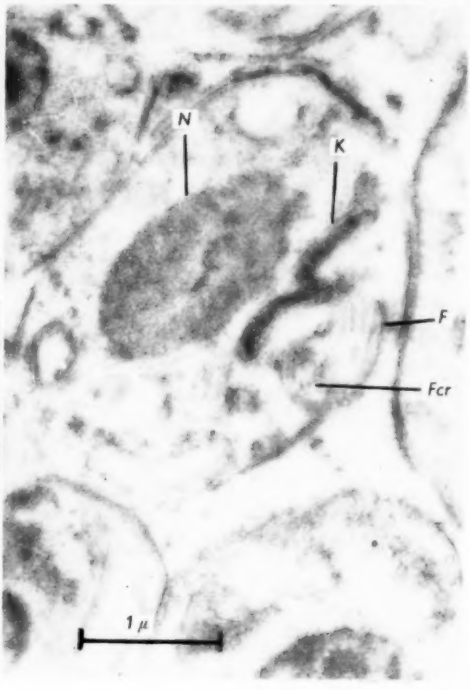
PLATE VI

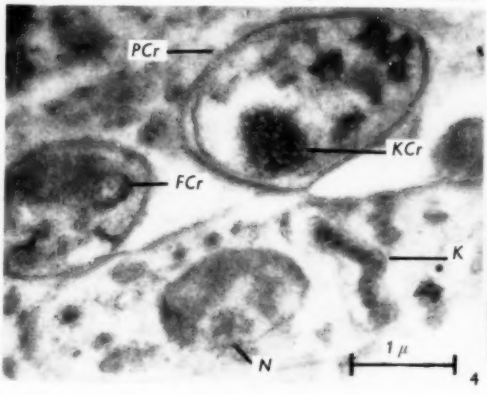
Schematic figure of the structure of *T. cruzi* in longitudinal and in cross-sections, as it appears during the different phases of the cycle. *L*, longitudinal section; *Cr*, cross-section; *M*, surface membrane, sheath; *P*, periplast; *N*, nucleus; *K*, kinetonucleus; *B*, basal corpuscles; *A*, axial fibres of flagellum; *V*, vacuole at posterior tip of trypanosome.

(*MS. received for publication* 21. VIII. 1956.—Ed.)









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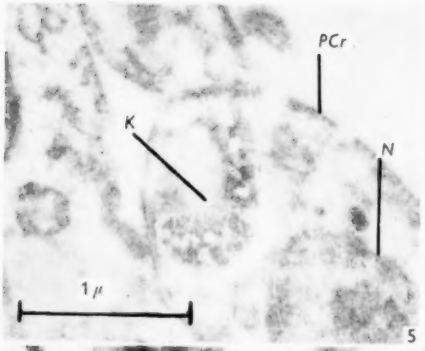
PCr
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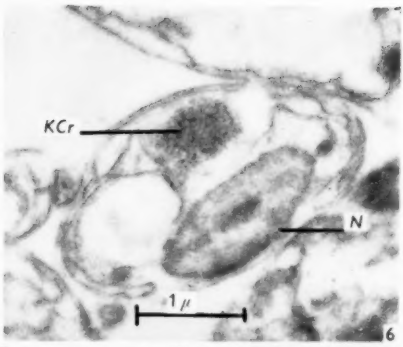
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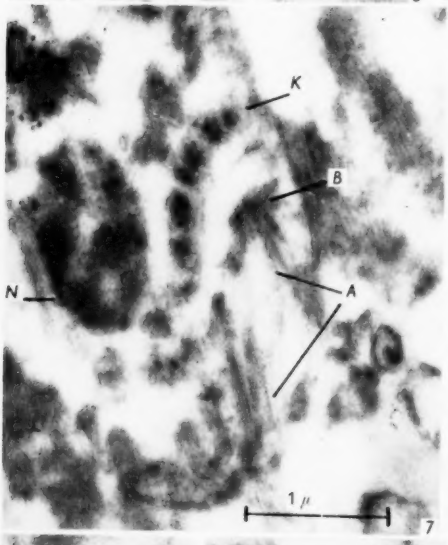
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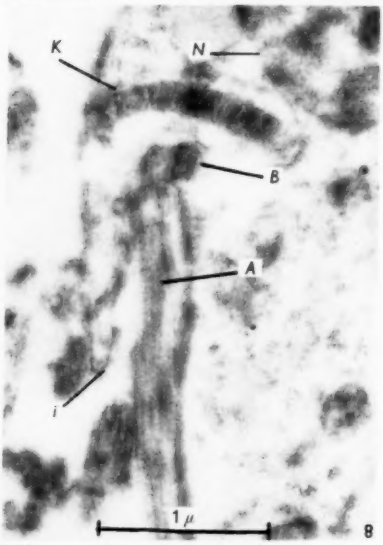
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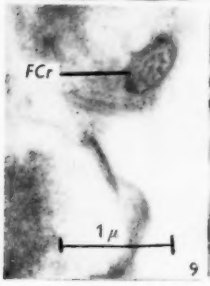
FCr
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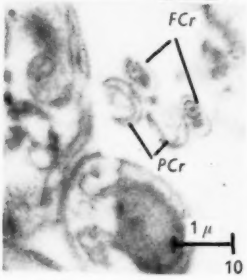
FCr
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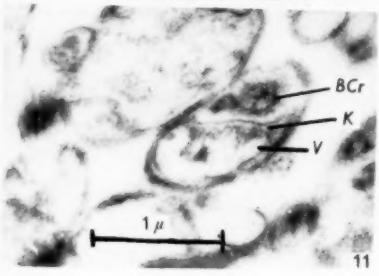
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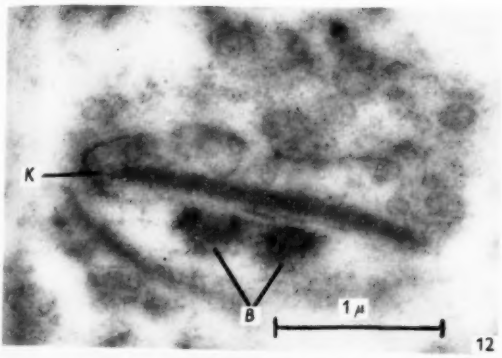

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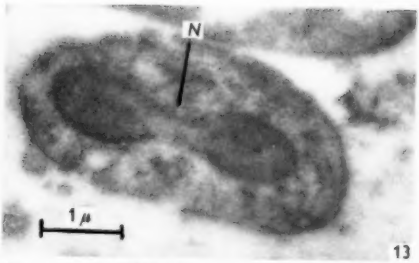

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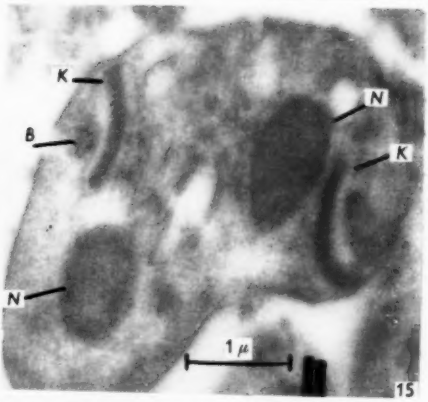

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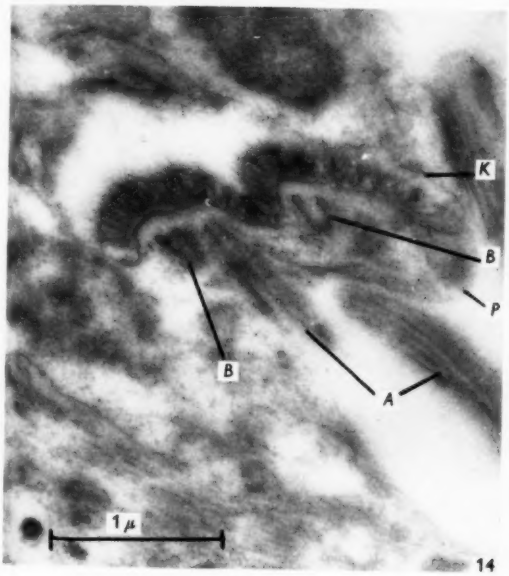

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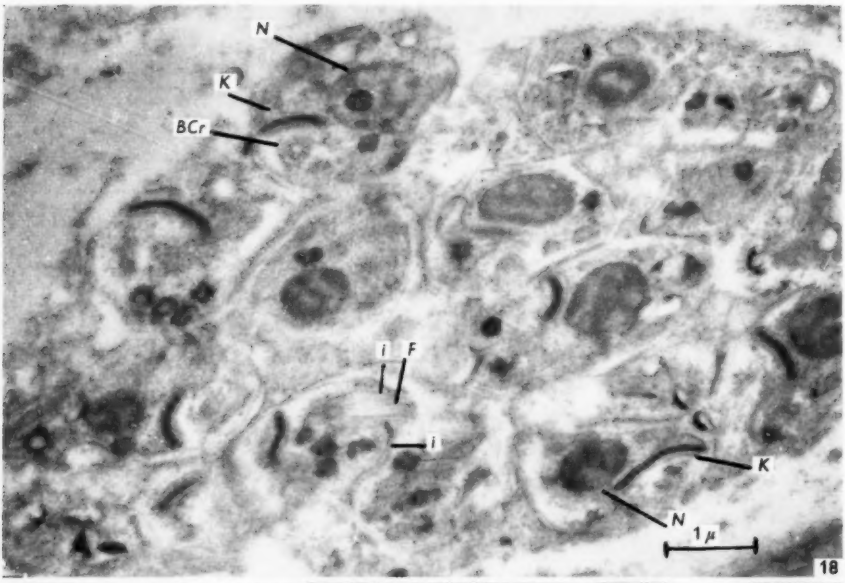
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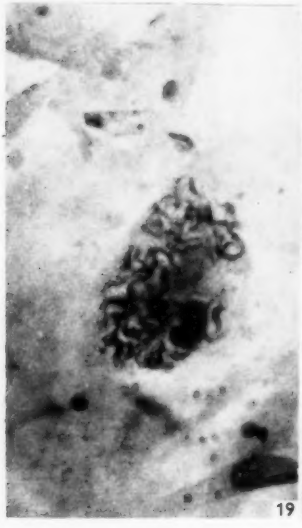
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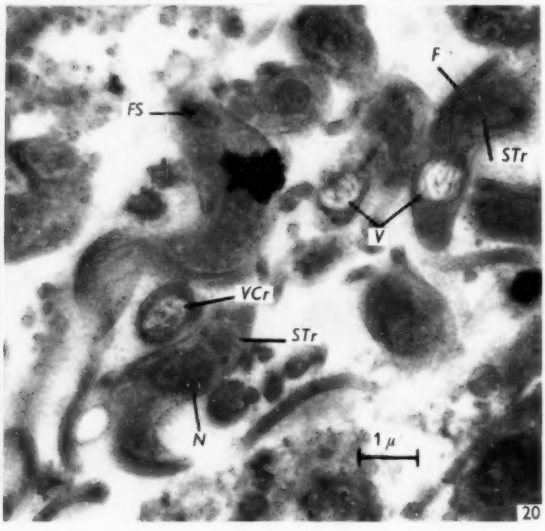
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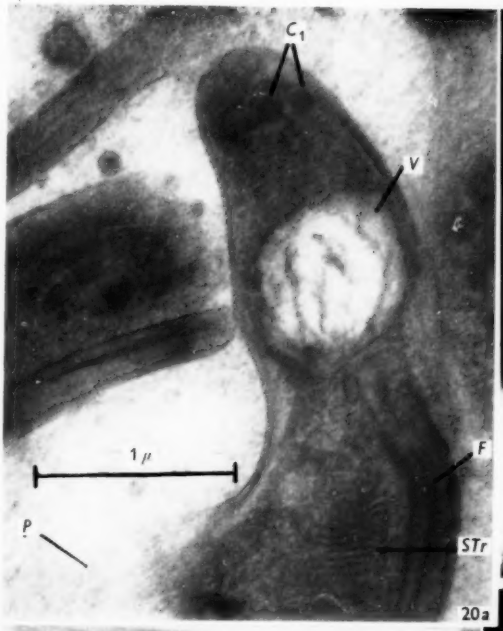

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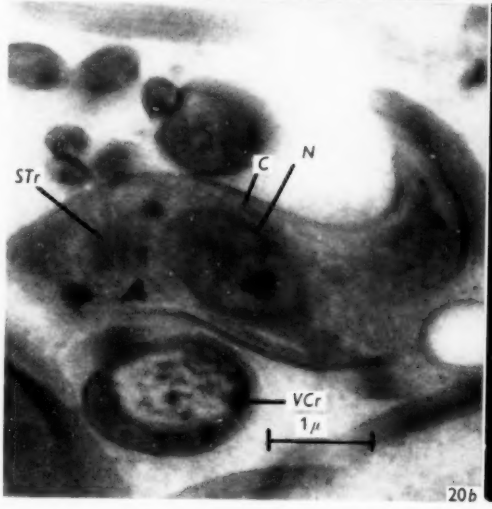

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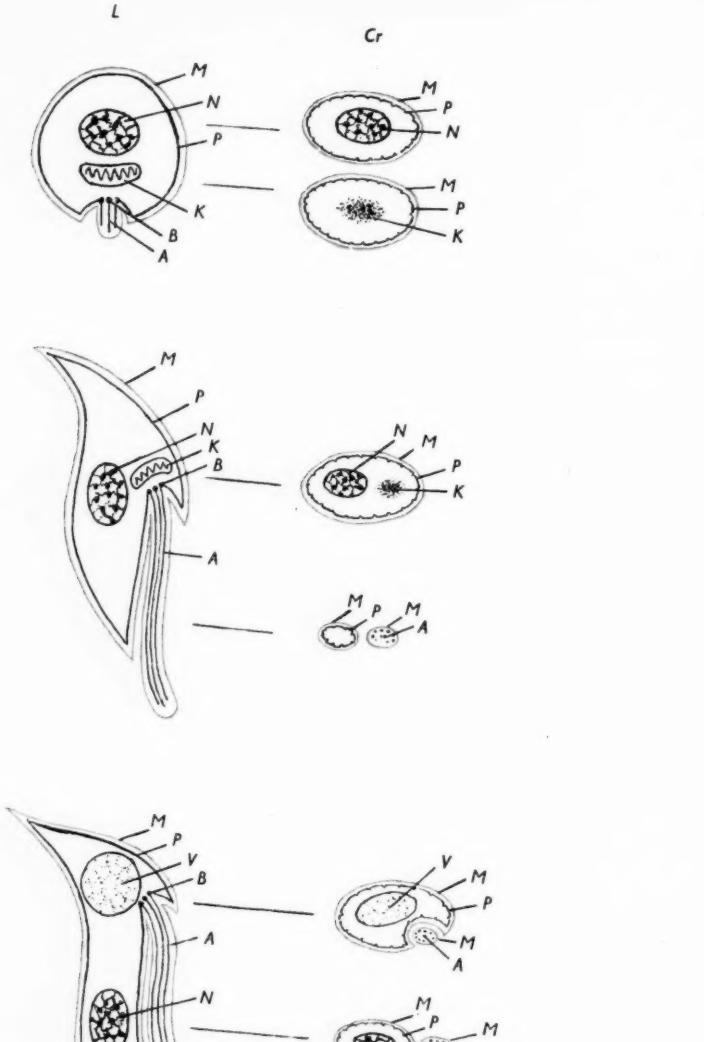
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A REVISION OF THE NEMATODE GENUS *METETERAKIS* KARVE, 1930

By WILLIAM G. INGLIS

Department of Zoology, British Museum (Natural History)

(With 22 Figures in the Text)

The genus *Meteterakis* was created for *M. govindi* sp.nov. by Karve (1930), who in discussing its affinities chose to compare it with *Heterakis* Dujardin, 1845. Baylis (1936) considered these two genera to be synonymous, a conclusion with which Koo (1939) later agreed. Subsequently, López-Neyra (1947) recognized that *Meteterakis* was more closely related to the genera placed by Travassos (1920) in the Heterakid subfamily Spinicaudinae, and referred it to this subfamily without comment. Apparently Skrjabin (1949) had not seen this paper and placed *M. govindi* in the genus *Ganguleterakis* Lane, 1914.

Thus there is some ambiguity about the status of *Meteterakis*, and recently an opportunity to clarify its position was made possible through the discovery of additional material in the collections at the British Museum (Natural History) and the London School of Hygiene and Tropical Medicine. This material appears to contain representatives of two new species and is sufficiently interesting to warrant an attempt at a complete revision of the genus. This has been carried out by a re-examination of as much as possible of the type material of species which appear, from their published descriptions, to belong to the genus. In so far as the main conclusions of the paper can be anticipated in these introductory remarks, it can be said that the genus is well founded, but its diagnosis has to be broadened to include eight distinct species which are here placed in it.

TECHNIQUE

During this study it has become obvious that most of the confusion that has arisen is due to poor and incomplete descriptions arising from faulty technique in the study of the specimens. As will be shown below, the possession of a gubernacular mass (a thickening of the walls of the cloaca) characterizes all the species in this genus, but this has often been overlooked, most probably because the specimens have been overcleared before study.

In this study all the specimens were first cleared in lactophenol and the details of the papillae on the tail and the form and extent of the gubernacular mass ascertained. The head can be studied only at this stage as its detailed structure later disappears with further clearing in creosote. In this medium the specimens become almost perfectly clear and all surface detail becomes invisible, but it enables the form of the spicules to be fully investigated. At this stage it is impossible to see the gubernacular mass in the smaller specimens, although it is still visible in the larger.

Family HETERAKIDAE Railliet & Henry, 1914

Subfamily SPINICAUDINAE Travassos, 1920

Although Yorke & Maplestone (1926, p. 23, footnote) and López-Neyra (1947) give the date of this subfamily as 1919, Travassos's original paper was clearly not published until 1920, for in the addendum to it is a reference to Stiles & Hassal's *Index Catalogue* which was not published until June of that year.

Genus **Meteterakis** Karve, 1930

The genus *Meteterakis* was proposed in 1930 for a single species, *M. govindi* Karve, 1930, but, as mentioned above, it was not considered a valid genus. As a result the type species appears to have been twice described subsequently as new, in different genera, and eight other species allied to it have been variously referred to *Africana*, *Ganguleterakis* and *Spinicauda*. This confusion has arisen through failure to appreciate the significance of differences in the gubernaculum and the tail. The following nominal species closely resemble, or appear to be identical with, *Meteterakis govindi*: *Africana howardi* Li, 1933; *A. mabuyae* Chakravarty, 1944; *A. varani* Maplestone, 1931; *Ganguleterakis triaculeatus* Kreis, 1933; *Meteterakis baylisi* sp.nov.; *M. louisi* sp.nov.; *Spinicauda bufonis* Yamaguti, 1935; *S. cophotis* Baylis, 1935; *S. japonica* Wilkie, 1930 and *S. longispiculata* Baylis, 1929; and all possess the following combination of characters. The male tail bears caudal alae supported by large fleshy papillae and a gubernacular mass is developed from the walls of the cloaca; in the females a valvular structure is developed from the anterior lip of the vulva, and in both sexes the excretory pore opens into a large, lobulated excretory vesicle. *Spinicauda* is characterized by the presence of a distinct gubernaculum, sessile papillae only on the tail, no caudal alae; *Africana* by caudal alae which are not supported by papillae, all of which are sessile. In both these genera the excretory system is simple and there is no flap over the vulva. *Ganguleterakis* is in my opinion not distinguishable from *Heterakis*, which differs from the *Meteterakis* group in the presence of broad caudal alae supported by long, thin papillae, no gubernaculum, no vulvar flap and a simple excretory system.

It seems desirable, therefore, to recognize this group of species as a distinct genus under the name *Meteterakis*. This has the following morphological features.

The structure of the head is typical of the family Heterakidae (Inglis, 1957), with three lips, each of which has the usual arrangement of pharyngeal teeth and an anterior cuticular flange which projects anterior to the mass of the lip, except in *M. triaculeatus* and *M. mabuyae*. The degree to which the lips are developed varies from species to species and is most easily understood from the figures.

The oesophagus is narrow with a relatively long anterior pharynx and a distinct, pear-shaped post-oesophageal bulb. The narrow portion of the oesophagus is lined throughout its length by thick refractive cuticle, which gives it a double appearance in optical section. The post-oesophageal bulb is entirely muscular and is valvulated. The nerve ring encircles the narrow part of the oesophagus anterior to the excretory pore which lies on the ventral surface and opens into a characteristic, large, lobulate excretory vesicle.

The intestine is swollen at its anterior end where it meets the oesophagus and is wider at this point than the post-oesophageal bulb.

The body bears narrow lateral alae which terminate at the level of the precloacal sucker in the male, and continue to the tip of the tail in the female.

Male

The tail, which is usually curled ventrally, has narrow caudal alae which, typically, are supported by three (exceptionally by four) pairs of large fleshy papillae. Of these two, or three, pairs lie about the level of the precloacal sucker and the remaining pair is lateral to the cloacal opening. In addition to these pairs there is a varying number of small sessile papillae which are very difficult to detect.

The precloacal sucker is rather small and has the heavily cuticularized rim characteristic of the family Heterakidae. The posterior lip of the cloacal opening is well cushioned and is covered by fine granulations. The tail ends in a fine, sharply pointed terminal spike.

The paired spicules vary considerably in appearance from species to species, but are always identical in structure and equal in length. The form of the spicules is the best character for distinguishing one species from another. A 'gubernacular mass', as I intend to call it, is always present, although the degree of development varies throughout the genus. It is a thickening of the cuticular lining of the cloacal walls, mainly dorsally and ventrally and less developed laterally. Its limits are difficult to establish and many authors have failed to see it, or if they have seen it have illustrated it incorrectly through not realizing its full extent and form.

Female

The vulva opens on the ventral surface, about the middle of the body, and is covered by a prominent flap developed from the anterior lip. It opens into a distinct vagina which immediately curls posteriorly to become a wider common uterine trunk. This continues posteriorly and splits into two parallel uteri which contain thin-shelled, oval eggs. The tail is long and pointed.

For diagnostic purposes these characters may be summed up thus:

Heterakidae: Spinicaudinae. Head with three lips; body generally bearing numerous small sessile papillae all over its surface and with narrow lateral alae in both sexes; pharynx long and oesophagus narrow; excretory pore opening into a large lobulate excretory vesicle. *Male*: spicules equal and identical; an indefinite gubernacular mass developed from the walls of the cloaca, except possibly in *M. triaculeatus*; narrow caudal alae supported by three, exceptionally by four, pairs of prominent fleshy papillae—two, or three, pairs at the level of the precloacal sucker and one pair lateral to the cloacal opening; a varying number of small sessile papillae also present; posterior lip of cloacal opening covered by small granulations. *Female*: vulva covered by a prominent flap developed from the anterior lip; vagina turns back as a common uterine trunk which continues straight posteriorly to split into two parallel uteri; tail long and pointed.

Hosts

Reptiles and amphibians.

Geographical distribution

India, Ceylon, China, Japan, East Indies and Burma.

Type species

Meteterakis govindi Karve, 1930.

In the descriptions that follow detailed measurements are given for the females, but without exception there do not appear to be any morphological characters by which it is possible to distinguish the females of one species from those of another. The form of the vagina, the vulva, the common uterine trunk and the relative lengths and positions of these parts are exactly the same in all the species. This is just what Maplestone (1932) and Madsen (1950) have reported in the genus *Heterakis* and it appears to be a characteristic of the Heterakidae.

There seem to be six named species and two hitherto undescribed that fall into this generic concept, as follows:

Meteterakis govindi Karve, 1930

Synonomy

Meteterakis govindi Karve, 1930—*Ann. trop. Med. Parasit.* **24**, 481–5, figs. 1–7; Walton, 1933, *J. Parasit.* **20**, 5.

? *Africana varani* Maplestone, 1931—*Rec. Indian Mus.* p. 33; Baylis, 1936, *The Fauna of British India, Nematoda*, I (London), pp. 149–50, fig. 73; Skrjabin, 1949, [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), p. 217, fig. 48; Skrjabin, 1951, [*List of Parasitic Nematodes*, II] (Moscow) (in Russian), p. 232.

Spinicauda bufonis Yamaguti, 1935—*Jap. J. Zool.* **6**, 388–9, figs. 1–2; Skrjabin, 1949, [*Nematode Parasites and the Illnesses Caused by Them*, I] (Moscow) (in Russian), p. 299, fig. 92; Skrjabin, 1951, [*List of Parasitic Nematodes*, II] (Moscow) (in Russian), p. 244.

Heterakis govindi, Baylis, 1936—*Fauna of British India, Nematodes*, I (London), pp. 126–7, fig. 51; Koo, 1939, *Lingnan Sci. J.* **18**, 148, table 5.

Ganguleterakis govindi, Skrjabin, 1949, [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), pp. 244–9, fig. 68; Skrjabin, 1951, [*List of Parasitic Nematodes*, II] (Moscow) (in Russian), p. 236.

Type host

Bufo melanostictus Schneider, 1799, rectum; Rangoon, Burma.

Other hosts and localities

Rectum of type host; Canton, China. Large intestine of type host; Tainan, Formosa (Yamaguti, *Spinicauda bufonis*). *Varanus bengalensis* (Daudin, 1802), Zoological Gardens, Calcutta. (Mapleston, *Africana varani*.)

Material studied

1♂, 2♀♀, '*Tree Frog*', Lingnan, China. British Museum (Natural History), registration number 1934.7.25.9–11.

1♀, type of *Africana varani*, Zoological Survey of India, registration number W. 1593/1.

Karve (1930) gave a very full and detailed description of this species, but he appears to have overcleared his specimens and so failed to note the gubernacular mass.

This species was also reported by Koo (1939), who gave a table of measurements, but no detailed description or figure of his specimens as he declared that they differed in minor details only from the description given by Karve. He does state that the 'caudal papillae of the male, except the three large pairs, have been found to be very variable in size, number and position. Therefore a definite description of groups as given by Karve is not of much value' and agrees with Baylis (1936) that this species should be considered a member of the genus *Heterakis*.

Male

The tail is very massively built when viewed from the lateral aspect and is flanked by the usual caudal alae supported by three fleshy papillae. In addition to these there are a varying number of small sessile papillae. In the specimen I have studied (Fig. 6) there are thirteen pairs on the tail and seven pairs on the ventral surface of the body anterior to the caudal alae. Of those on the tail two pairs are suctorial, one pair is pre-cloacal, four pairs are para-cloacal and six pairs are post-cloacal. Three pairs of these post-cloacal papillae are ventral, one pair is lateral and two pairs are dorso-lateral in position.

Karve describes seventeen pairs, 'some of them very minute and difficult to detect', of which three are fleshy and appear to be consistent throughout the species, while the others may vary or be overlooked. Yamaguti (1935), in describing *Spinicauda bufonis*, also mentions, and figures, these three papillae and describes the others as being small and variable.

The spicules are very thin and are slightly alate at the posterior end. The alae are borne on extremely narrow continuations of the main mass of the spicules, and are partly masked by the gubernacular mass. It is probable that Karve failed to see the alae and described the backing portion only. The anterior ends are turned slightly ventrally and are widely cup-shaped (Fig. 7).

There is the usual gubernacular mass which is approximately 0·11 mm. long. It is represented in Yamaguti's figure of *S. bufonis* as a *ventral* mass which is labelled 'gub' (gubernaculum?), but is not referred to in the description except inferentially in the statement that *S. bufonis* does not differ from *S. japonicum* in any important detail other than in the detailed structure of the spicules. The mass figured by Yamaguti only represents part of the extensive body of cuticularization actually present.

Female

The body reaches its full width about the level of the vulva which is covered by the typical flap. This vulva opens into a looped vagina, about 0·23 mm. long, which turns back immediately as a common uterine trunk. This continues posteriorly for 1·1–1·3 mm., where it separates into two parallel uteri. The eggs are 68–76 × 41–46μ in size. The tail is long and pointed and the lips of the anus are prominently raised.

Discussion

Spinicauda bufonis Yamaguti, 1935, seems to be indistinguishable from *Meteterakis govindi*.

Unfortunately, the only specimen, and type, of *Africana varani* Maplestone, 1931 which I have been able to study is a female which, from the systematic viewpoint, is of little value. However, Maplestone's original description (1931), although insufficient for complete comparison, leaves me in little doubt that this, also, is a synonym of *Meteterakis govindi*.

Table 1. Meteterakis govindi (*measurements in mm.*)

	Male	Female
Length of body	4·0–5·4	4·6–7·2
Breadth of body	0·20–0·29	0·25–0·37
Total length of oesophagus	0·67–1·00	0·93–1·05
Length of post-oes. bulb.	0·19–0·30	0·19–0·30
Breadth of post-oes. bulb.	0·11–0·19	0·14–0·19
Length of pharynx	0·043–0·061	0·045–0·073
Diameter of head	0·035–0·062	0·035–0·058
Nerve ring from anterior end	0·25–0·36	0·28–0·30
Excretory pore from anterior end	0·50–0·56	0·51–0·54
Length of spicules	0·18–0·27	—
Length of tail	0·13–0·23	0·24–0·35
Diameter of sucker	0·038–0·050	—
Distance from sucker to cloaca	0·050–0·069	—
Vulva from the anterior end	—	2·2–3·2

Meteterakis baylisi sp.nov.

Type host

Ceratophora stoddarti Gray, 1834, not known: Hagkala, Ceylon.

Material studied

The type specimens in the collections of the British Museum (Natural History), viz. holotype ♂, 1935.4.16.76; allotype ♀, 1935.4.16.77; paratypes 3 ♂♂, 4 ♀♀, 1935.4.16.78–85.

These specimens were obtained by Dr Malcolm Smith from a specimen which had been presented to the British Museum (Natural History) by Mr W. G. Freeman. They had been provisionally identified as *Spinicauda* sp. Their condition is not good, but sufficient detail can be made out to suggest that they represent a new species.

I have been unable to make out the structure of the head due to the poor condition of the specimens, but it appears probable, in view of the other similarities, that it will not differ in any great respect from that in the other species of the genus. The body is strongly curved ventrally at both ends and because of this, and the massive construction of the spicules, I have been unable to obtain a ventral view of the male tail. The oesophagus is much curled within the body, most probably due to the effect of fixation.

Male

The tail is strongly curved ventrally and bears the usual caudal alae and three fleshy papillae typical of the genus. There is a varying number of small sessile papillae, the arrangement of which is best understood from the figure (Fig. 8), but there are possibly many more which I have not seen due to the condition of the specimens.

The spicules are massive with the anterior ends strongly curved ventrally. They narrow rapidly about half-way along their length, but develop large alae at the same level. They are tessellate over their entire surface, except for the alae which are very poorly cuticularized and are transparent. The posterior ends are very characteristic, having a cap-like development on the tips. The anterior ends are cupped for the reception of the spicular muscles (Fig. 1). A strongly developed gubernacular mass is present.

Female

The vulva has the usual well-developed flap on the anterior lip and opens into the typical arrangement of vagina and common uterine trunk. The latter runs posteriorly for 1·0–1·3 mm. before separating into two parallel uteri. The eggs are 58–64 × 37–42μ.

Discussion

This species approaches *Meteterakis longispiculata* in general appearance, but is easily distinguished from it by the form of the spicules. The capitate ends of these are most distinctive and are easily recognized.

Meteterakis cophotis (Baylis, 1935)

Synonymy

Spinicauda cophotis Baylis, 1935—*Ann. Mag. nat. Hist.* (10), **16**, 187–90, fig. 1; Skrjabin, 1949, [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), pp. 300–3, fig. 94; Skrjabin, 1951, [*List of Parasitic Nematodes*, II], (Moscow) (in Russian), p. 244.

Type host

Cophotis ceylanica Peters, 1861, not known: Gammaduwa, Ceylon.

Other hosts and localities

Lyriocephalus scutatus (Linnaeus, 1758): Gammaduwa, Ceylon.

Material studied

7 ♂♂, 8 ♀♀, co-types and paratypes, British Museum (Natural History), registration numbers 1935.9.12.23–37, of which I here select 1♂, 1935.9.12.23 as lectotype.

This species was described by Baylis (1935) from material collected by Mr

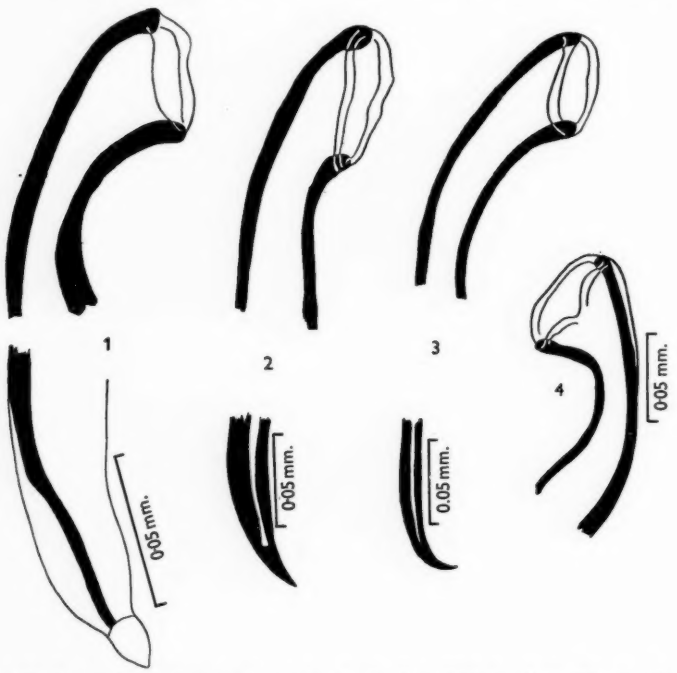


Fig. 1. Detail of anterior and posterior ends of spicules of *M. baylisi*. Fig. 2. The same for *M. japonica*. Fig. 3. The same for *M. longispiculata*. Fig. 4. Detail of anterior end of spicules in *M. cophotis*.

Table 2. Meteterakis baylisi (*measurements in mm.*)

	Male	Female
Length of body	3·0–3·7	3·6–4·2
Breadth of body	0·21–0·28	0·24–0·31
Total length of oesophagus	0·72–0·81	0·66–0·82
Length of post-oes. bulb.	0·13–0·16	0·16–0·19
Breadth of post-oes. bulb.	0·12–0·16	0·15–0·17
Length of pharynx	0·056–0·058	0·049–0·052
Diameter of head	0·074–0·054	0·045–0·052
Nerve ring from anterior end	0·34–0·37	0·36–0·39
Excretory pore from anterior end	0·51–0·55	0·49–0·51
Length of spicules	0·42–0·45	—
Length of tail	0·19–0·23	0·22–0·26
Diameter of sucker	0·032–0·047	—
Distance from sucker to cloaca	0·012–0·015	—
Vulva from anterior end	—	1·7–2·2

D. R. R. Burt, Ceylon University College, from both the hosts given above. Baylis states: 'The specimens from the former host (*Cophotis ceylanica*) are slightly larger and are taken as the types.'

Male

The tail is very similar to that of *M. longispiculata*; it is similarly curled ventrally and appears disproportionately large dorso-ventrally. The sucker is relatively small and the tail has the usual large fleshy papillae supporting the caudal alae. Anterior to the sucker, there are at least seven pairs of small sessile papillae (Baylis

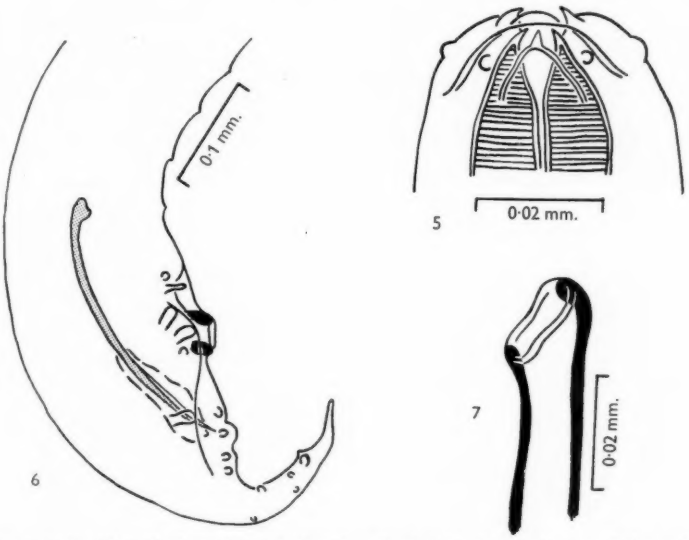


Fig. 5. *M. govindi.* Dorsal view of head, showing the internal structure and the extent of the anterior cuticular flange. Fig. 6. *M. govindi.* Male tail from the right. Fig. 7. *M. govindi.* Detail of the structure of the anterior ends of the spicules.

says five (?)) and two pairs of sessile ventro-lateral papillae. One or two pairs of sessile papillae flank the pre-cloacal sucker and there are two pairs, one of which is very prominent with a finger-like tip, lying on the anterior lip of the cloaca. There are five pairs of sessile papillae post-cloacal, of which two pairs are dorso-lateral, two pairs ventro-lateral and one, the terminal pair, ventral (Fig. 10).

The spicules are large, equal and identical in form and structure, with the anterior ends strongly curved ventrally (Fig. 4). About half-way along their length they narrow rapidly (Fig. 10) and develop non-tessellate alae at the same level. These alae continue, narrowing, to the posterior ends of the spicules which are sharply pointed. The usual gubernacular mass, which is very well developed, is present and the posterior lip of the cloacal opening is cushioned.

Female

The usual flap overhangs the vulva, but the sudden constriction immediately behind the vulva reported by Baylis is not present in all the specimens and appears

to be an artifact. The common uterine trunk is about 1·4 mm. long and the eggs are 68–76 × 42–48 μ in size.

Discussion

As Baylis pointed out (1935), this species is extremely similar to *M. longispiculata*, which is re-described below (p. 22). He considers that 'the number and arrangement of the caudal papillae of the male, and the form of the vulva and vagina in the female, appear to be distinguishing characters.' The form of the vulva in both these species is in fact the same and the form of the vagina is probably more an indication of sexual maturity than of specific distinctness. The number of small sessile papillae on the tail appears to be a variable character of no specific value. The spicules in this species have poorly cuticularized alae, while in *M. longispiculata* this portion is strongly tessellated. The spicules are in all other respects identical, and while this difference is most probably due only to variation within one species, I prefer, like Baylis, to treat this species, *M. cophotis*, as distinct from *M. longispiculata* until more material is available for study.

Table 3. Meteterakis cophotis (*measurements in mm.*)

	Male	Female
Length of body	6·4–8·0	7·3–9·0
Breadth of body	0·35–0·37	0·24–0·37
Total length of oesophagus	0·9–1·0	1·0–1·2
Length of post-oes. bulb.	0·18–0·21	0·20–0·22
Breadth of post-oes. bulb.	0·15–0·17	0·19–0·21
Length of pharynx	0·07–0·08	0·07–0·08
Diameter of head	0·056–0·061	0·058–0·068
Nerve ring from anterior end	0·30–0·34	0·33–0·36
Excretory pore from anterior end	0·50–0·55	0·52–0·57
Length of spicules	0·64–0·81	—
Length of tail	0·29–0·38	0·40–0·55
Diameter of sucker	0·06–0·07	—
Distance from sucker to cloaca	0·067–0·074	—
Vulva from anterior end	—	3·0–4·3

Meteterakis japonica (Wilkie, 1930)

Synonymy

Spinicauda japonica Wilkie, 1930—*Ann. Mag. nat. Hist.* (10), **6**, 612–13, fig. 4; Hsü, 1933, *Parasitology*, **24**, 517–18, figs. 11–14 and 21; Walton, 1933, *J. Parasit.* **20**, 5; Yamaguti, 1935, *Jap. J. Zool.* **6**, 387–8; Yamaguti, 1941, *Jap. J. Zool.* **9**, fig. 10; Kung & Wu, 1945, *Sinensia*, **16**, 79; Skrjabin, 1949, [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), pp. 303–4, figs. 96–97; Skrjabin, 1951, [*List of Parasitic Nematodes*, II] (Moscow) (in Russian), p. 232.

Africana howardi Li, 1933, *Chinese Med. J.* **47**, 1322–4, fig. 24029; Skrjabin, 1949, [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), pp. 213–14, fig. 47; Skrjabin, 1951, [*List of Parasitic Nematodes*, II] (Moscow) (in Russian), p. 232.

Type host

'*Bull Frog*', not known: Japan.

Other hosts and localities

Bufo bufo japonicus Schlegel, 1838, rectum: Hochow, Szechwan, China. Large intestine: Schegel, Siga Prefecture; and small intestine: Sirahama, Wakayama, Prefecture, Japan.

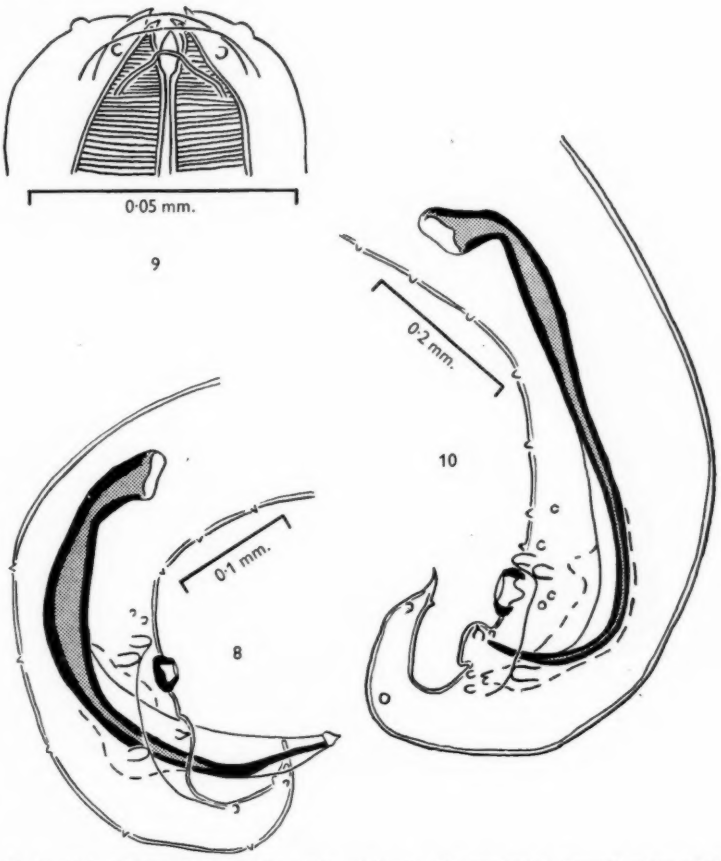


Fig. 8. *M. baylisi.* Male tail from the right. Fig. 9. *M. cophotis.* Dorsal view of the head, showing the internal structure. Fig. 10. *M. cophotis.* Male tail from the left.

Bufo bufo gargarizans Cantor, 1842, rectum and small intestine: Pehpei, Szechwan, China and Shanghai.

Bufo bufo formosus Boulenger, 1883, small intestine: Shanghai.

Material studied

4 ♂♂, 5 ♀♀, co-types, British Museum (Natural History), registration numbers 1931.3.12.27–36. I select 1 ♂, 1931.3.12.27 as lectotype.

This species was first described by Wilkie (1930) from material collected by Professor A. S. Pearse, Duke University, North Carolina, U.S.A., during a visit to

Keiogijuku University, Tokyo, Japan, and has since been re-described by Hsü (1933), Yamaguti (1935, 1941) and Kung & Wu (1945). Wilkie's description is accompanied by a rather poor figure, in which the spicules have been displaced by attempts to roll the specimen so as to obtain a ventral view of the tail. This manipulation is extremely difficult to carry out because of the shape of the spicules, and I have not been successful with any of the specimens I have studied.

Morphology

The body is short and stout, especially when contrasted with *M. longispiculata*, and is bent ventrally at both the anterior and posterior ends. In all other respects the morphology of this species conforms to the generic pattern.

Male

The three pairs of large fleshy papillae typical of the genus are present. There are in addition a varying number of smaller sessile papillae the arrangement of which is most easily understood by reference to Fig. 12.

Wilkie describes thirteen pairs, Hsü twenty pairs, Yamaguti twenty pairs, and the maximum number I have been able to determine is also twenty pairs. As Yamaguti (1935) has pointed out, Wilkie is in error when he states that there are three pairs of large fleshy papillae anterior to the cloacal opening, as he only figures two pairs, and that is all I have been able to find in his specimens.

The spicules are massive with the anterior ends bent ventrally. They are identical in structure and are non-alate. The anterior ends are deeply cup-shaped (Fig. 2), and the posterior ends are sharply pointed (Fig. 2). The entire surface is tessellate. There is a gubernacular mass present, developed in the usual way from the walls of the cloaca. Wilkie figures this as a gubernaculum with a definite outline, while Hsü does not show it in his figure of the male tail, although he gives the length of the gubernaculum as 0·020 mm. (Hsü, fig. 14). Yamaguti (1941) gives a photograph of the male tail, in which it is difficult to make out the spicules and it shows nothing of the gubernacular mass.

Female

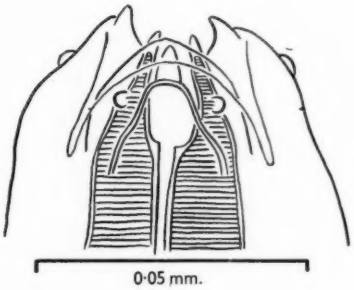
The body is longer and thinner than that of the male. The vulva is typical and there are frequently swellings of the cuticle anterior and posterior to the vulva, which are most probably due to the action of the male precloacal sucker during copulation. The vagina is 0·5–0·7 mm. long and the common uterine trunk is 1·0–1·4 mm. long. The eggs are 76–90 × 40–50 μ. The lips of the anus are prominent and the tail is long and evenly pointed.

Discussion

This species is easily separated from *M. longispiculata* which it closely resembles by the non-alate form of the spicules and the relative stoutness of the body.

Hsü (1933) and Kung & Wu (1945) agree in their descriptions, and state that the differences between their material and the description given by Wilkie (1930)

appear to be beyond the limits of variation, but also state that they await re-examination of the type material before erecting a new species. My re-examination of the type material shows that Wilkie's description is at fault and that the material, corresponds fully with the descriptions given by the other authors.



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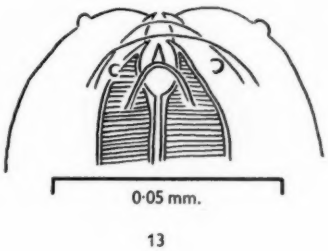

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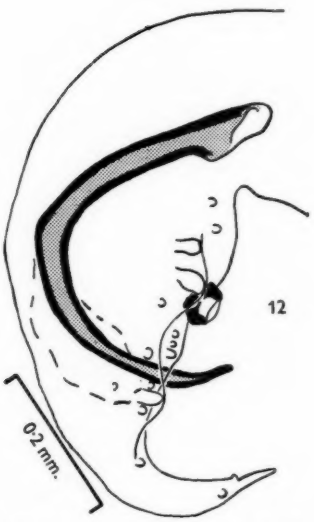

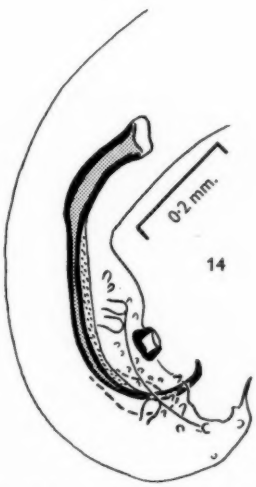

Fig. 11. *M. japonica.* Dorsal view of the head, showing internal structure. Fig. 12. *M. japonica.* Male tail from the right. Fig. 13. *M. longispiculata.* Dorsal view of head showing detail of the internal structure. Fig. 14. *M. longispiculata.* Male tail from the right.

Specimens from *Bufo bufo japonicus* were described as new by Li (1933) under the name *Africana howardi*. He failed to see the gubernacular mass, but his figures are so very good that there can be no doubt that his specimens are actually *Meteterakis japonica*.

Table 4. Meteterakis japonica (*measurements in mm.*)

	Male	Female
Length of body	3·0–6·1	4·6–7·7
Breadth of body	0·21–0·38	0·26–0·42
Total length of oesophagus	0·77–1·18	0·75–1·25
Length of post-oes. bulb.	0·16–0·22	0·16–0·23
Breadth of post-oes. bulb.	0·09–0·16	0·16–0·21
Length of pharynx	0·06–0·08	0·06–0·09
Diameter of head	0·037–0·041	0·042–0·051
Nerve ring from anterior end	0·26–0·37	0·25–0·37
Excretory pore from anterior end	0·45–0·60	0·38–0·58
Length of spicules	0·46–0·69	—
Length of tail	0·18–0·30	0·34–0·55
Diameter of sucker	0·04–0·06	—
Distance from sucker to cloaca	0·021–0·027	—
Vulva from anterior end	—	1·4–2·9

Meteterakis longispiculata (Baylis, 1929)

Synonymy

Spinicauda longispiculata Baylis, 1929—*Parasitology*, **21**, 256–7, fig. 1; Skrjabin, 1949, [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), pp. 304–7, fig. 68; Skrjabin, 1951, [*List of Parasitic Nematodes*, II] (Moscow) (in Russian), p. 246.

Type host

Gecko gecko (Linnaeus, 1758), intestine: Samarang, Java.

Material studied

3 ♂♂, 3 ♀♀, co-types, British Museum (Natural History), registration numbers 1929.10.25.1–6. I select 1 ♂, 1929.10.25.1 as lectotype.

This species was described by Baylis (1929) from material collected by Dr E. Jacobson in Java.

Male

The tail, which is always curled ventrally, is very deep dorso-ventrally and appears to be disproportionately large. The usual alae and fleshy papillae are present, together with a large and variable number of small sessile papillae. These are mainly ventral or subventral in position. The number of post-cloacal papillae appears to be constant at eight pairs of which one pair is dorso-lateral and the other pairs are ventral or ventro-lateral. The maximum number of papillae which I have seen is twenty, in addition to the large fleshy papillae. The arrangement of these papillae is best understood from the figure (Fig. 14).

The spicules are large, equal and identical. They have very strongly expanded anterior ends (Fig. 3) which are bent in a characteristic manner towards the ventral surface of the body. Posteriorly they narrow rapidly and bear rather heavy alae (Fig. 14). They are tessellated for their entire length, including the alate portion. The usual gubernacular mass is very prominent.

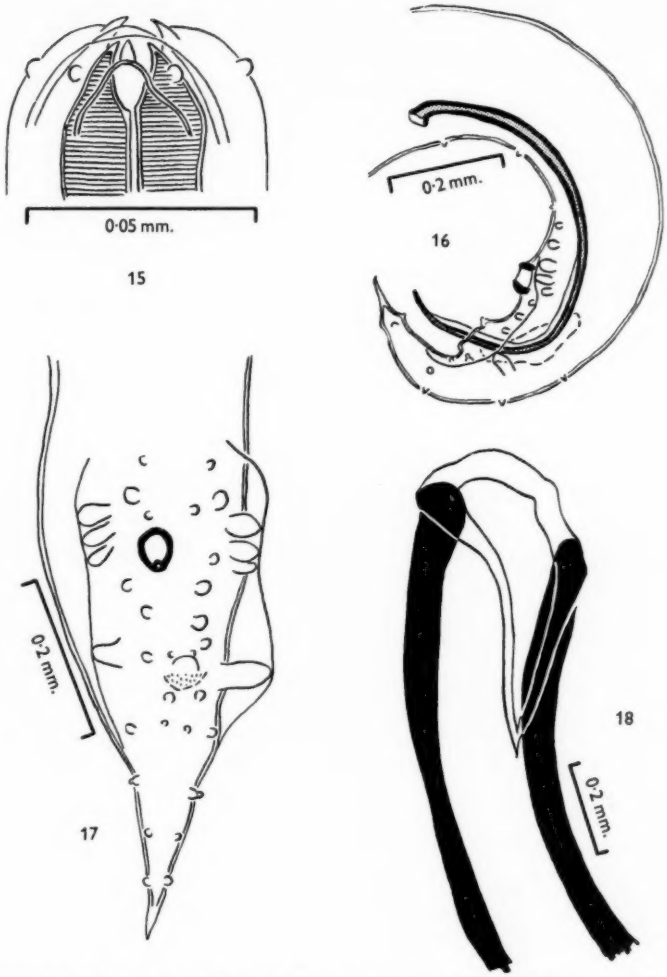

Fig. 15. *M. louisi.* Dorsal view of head. Fig. 16. *M. louisi.* Male tail from the left. Fig. 17. *M. louisi.* Ventral view of male tail. Fig. 18. *M. louisi.* Detail of anterior ends of spicules.

Female

In some specimens there are cuticular swellings anterior and posterior to the vulva, which can be attributed to the action of the male sucker. The common uterine trunk is about 1·3 mm. long, but because of the state of the specimens it is difficult to be sure of its limits. The eggs are 73–84 × 44–48 μ. Baylis reports that one female he studied contained eggs as large as 0·1125 × 0·1155 mm.

The lips of the anus, especially the rear ones, are prominently raised.

Discussion

See above (p. 18) under *M. cophotis.*

Table 5. Meteterakis longispiculata (*measurements in mm.*)

	Male	Female
Length of body	7·0–7·5	7·5–7·8
Breadth of body	0·28–0·35	0·30–0·39
Total length of oesophagus	1·03–1·30	1·20–1·46
Length of post-oes. bulb.	0·20–0·23	0·22–0·26
Breadth of post-oes. bulb.	0·15–0·20	0·17–0·21
Length of pharynx	0·08–0·10	0·08–0·10
Diameter of head	0·041–0·053	0·043–0·061
Nerve ring from anterior end	0·38–0·41	0·40–0·45
Excretory pore from anterior end	0·68–0·76	0·73–0·80
Length of spicules	0·63–0·68	—
Length of tail	0·27–0·31	0·34–0·38
Diameter of sucker	0·050–0·063	—
Distance from sucker to cloaca	0·072–0·083	—
Vulva from anterior end	—	3·0–3·5

Meteterakis louisi sp.nov.

Type host

'*Tree Lizard*', stomach: India, no more precise locality known.

Material studied

7 ♂♂, 6 ♀♀, London School of Hygiene and Tropical Medicine, Helminthological Collection, number 879. Holotype ♂ and allotype ♀ have been placed separately in tubes.

These specimens were collected by Mr W. Ware in India and were registered under the name *Heterakis* sp. Their condition is fairly good and it is possible to give a full description of their morphology.

Male

The tail is strongly curled ventrally and the arrangement of the large fleshy papillae is atypical. There are four pairs, of which three pairs are at the level of the precloacal sucker, and one is at the level of the cloacal opening. Of the anterior group the anterior two pairs are larger than the third more posterior pair (Figs. 16, 17). There are, in addition, about fourteen or fifteen pairs of smaller sessile papillae, the arrangement of which is best appreciated from the figures.

The spicules are identical in form and structure. They are very long and thin with narrow alae developed for about the last half of their lengths. They end posteriorly in fine points and are strongly cupped anteriorly, the cup is heavily incised on one side (Fig. 18). The gubernacular mass is very indistinct and is indicated only approximately in the figure.

Female

All the females are fully gravid and are therefore swollen by the eggs which they contain. The usual flap is present over the vulva and the common uterine trunk runs back to a point 0·9–1·2 mm. behind it before splitting into the usual parallel uteri. The eggs are 66–74 × 40–48 μ in size.

Discussion

This species is distinct from all the others included in the genus *Meteterakis* in the size and form of the spicules and in the arrangement of the fleshy papillae of the male tail. In all the other species of the genus there are only two pairs of fleshy papillae lateral to the pre-cloacal sucker, while in this species there are three. In other respects it is a typical member of the genus.

Table 6. Meteterakis louisi (*measurements in mm.*)

	Male	Female
Length of body	5·0–7·4	6·5–7·6
Breadth of body	0·37–0·45	0·42–0·46
Total length of oesophagus	0·98–1·12	1·12–1·16
Length of post-oes. bulb.	0·19–0·22	0·21–0·22
Breadth of post-oes. bulb.	0·16–0·18	0·18–0·20
Length of pharynx	0·073–0·079	0·076–0·081
Diameter of head	0·051–0·058	0·061–0·066
Nerve ring from anterior end	0·34–0·36	0·36–0·42
Excretory pore from anterior end	0·59–0·61	0·58–0·64
Length of spicules	0·97–1·10	—
Length of tail	0·26–0·34	0·31–0·36
Diameter of sucker	0·053–0·059	—
Distance from sucker to cloaca	0·096–0·116	—
Vulva from anterior end	—	2·3–2·9

Meteterakis mabuyi (Chakravarty, 1944)

Synonymy

Africana mabuyae Chakravarty, 1944—*J. Dep. Sci., Calcutta Univ.*, N.S., **1**, 70–3, fig. 1; Skrjabin, 1949, [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), pp. 217; Skrjabin, 1951, [*List of Parasitic Nematodes*, II] (Moscow) (in Russian), p. 232.

Type host

Mabuya carinata Schneider (1801), intestine: Calcutta, India.

Material examined

1 ♀, type, Zoological Survey of India, registration number W. 3503/1.

This species was described by Chakravarty (1944) from one male and one female specimen. Unfortunately I have only been able to study the female which is registered in the collections of the Zoological Survey of India as type. It is in an extremely poor state and only the anterior part of the body is in a suitable condition for examination. This has allowed me to note that the structure of the head is identical with that of *M. triaculeata* (see below, p. 28) in that the anterior flange does not project beyond the main mass of the lips.

The following description is that given by Chakravarty (1944), slightly condensed:

The worms are small without lateral alae. The structure of the oesophagus is typical of the genus.

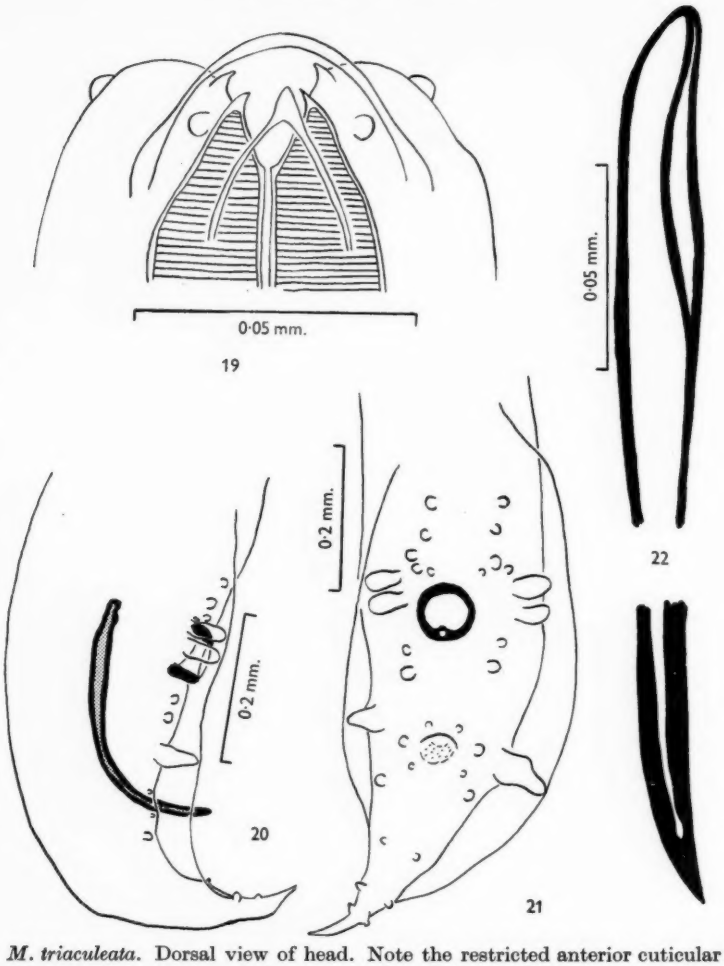

Fig. 19. *M. triaculeata.* Dorsal view of head. Note the restricted anterior cuticular flange. Fig. 20. *M. triaculeata.* Male tail from the right. Note that in rolling this specimen the para-cloacal large fleshy papilla has been displaced and appears to lie anterior to the cloacal opening. Fig. 21. *M. triaculeata.* Ventral view of male tail. Fig. 22. *M. triaculeata.* Details of spicule. Note the structure of the anterior end.

Male

The tail is curled ventrally and is flanked by narrow caudal alae extending from the anterior border of the pre-cloacal sucker to a little posterior to the cloacal opening. There are twenty pairs of caudal papillae, three pairs anterior to the pre-cloacal sucker, two pairs lateral to the sucker, four pairs between it and the cloacal opening, three pairs para-cloacal and the rest are post-cloacal in position.

Female

The vulva is prominent (? valve on anterior lip), opening into the vagina which runs posteriorly. The tail tapers evenly to a fine point. The eggs are 75–100 × 50 μ.

Discussion

It seems possible that this species is identical with *M. triaculeata* (described below), which it resembles in the atypical form of the head and, as far as I can make out, in the length and structure of the spicules. I think it probable that Chakravarty's figure of the male tail, in which the cloacal and pre-cloacal papillae are shown as identical in size and structure, i.e. all fleshy, is inaccurate, but as I have not been able to examine the male specimen I prefer to leave this species, provisionally, distinct.

Table 7. Meteterakis mabuyi (*measurements in mm. from Chakravarty*, 1944)

	Male	Female
Length of body	4·7	7·025
Breadth of body	0·25	0·35
Total length of oesophagus	0·75	0·975
Excretory pore from anterior end	0·27	0·55
Length of tail	0·15	0·25
Length of spicules	0·3	—
Vulva from anterior end	—	2·6

Meteterakis triaculeata (Kreis, 1933)

Synonymy

Ganguleterakis triaculeatus Kreis, 1933—*Z. Parasitenk.* **6**, 332–8, figs. 1–8; Kreis, 1940, *Zbl. Bakt.* **145**, 164; Skrjabin, 1949, [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), pp. 268–78, fig. 80; Skrjabin, 1951, [*List of Parasitic Nematodes*, II] (Moscow) (in Russian), p. 239.

Type host

Corucia zebrata Gray, 1855, intestine: Solomon Islands.

Material studied

1 ♂, 3 ♀♀, co-types. Naturhistorisches Museum, Basel; registration number 11-a. I select the male specimen as lectotype.

This species has been recorded once, by Kreis (1933) in the original description. This description is excellent, but the dimensions are given in the form of Cobb's formula which reduces their value to the parasitologist considerably.

Male

The three large fleshy papillae typical of the genus are present on the tail as is a varying number of smaller sessile papillae, the arrangement of which is most easily understood from the figures (Figs. 20, 21). Kreis overlooked these smaller papillae, which he has not included in his figures of this species. I have been unable to find any trace of a gubernacular mass in this species, and it seems most unlikely that I have overlooked it.

The spicules are short and simple. They do not turn ventrally at the anterior

ends and are sharply pointed at the posterior ends (Fig. 22). The anterior ends are different from those of all the other species in the genus in that they have lateral grooves rather than cupped ends for the reception of the spicular muscles.

Female

All the females are gravid and swollen. The vulva is covered by the usual flap, the common uterine trunk is 1·1–1·4 mm. long and the eggs are 62–74 × 37–41 μ. The posterior lip of the anus is raised and the tail is relatively short and stout.

Discussion

This species differs from all the other species I refer to this genus in the non-alate form of the small spicules, the lack of a gubernacular mass and in the degree of development of the anterior cuticular flange of the lips (Fig. 19). It is possible that it is sufficiently distinct to warrant the erection of a new genus for its reception, but due to the small number of species in the genus and the restricted amount of material it seems advisable to leave it in the genus, at least for the time being.

Table 8. Meteterakis triaculeata (*measurements in mm.*)

	Male	Female
Length of body	9·4	8·2–9·8
Breadth of body	0·42	0·49–0·53
Total length of oesophagus	3·11	2·90–3·16
Length of post-oes. bulb.	0·47	0·42–0·45
Breadth of post-oes. bulb.	0·38	0·31–0·39
Length of pharynx	0·21	0·22–0·23
Diameter of head	0·098	0·11–0·12
Nerve ring from anterior end	0·39	0·40–0·41
Excretory pore from anterior end	1·25	1·27–1·31
Length of spicules	0·38	—
Length of tail	0·32	0·42–0·43
Diameter of sucker	0·87	—
Distance from sucker to cloaca	0·13	—
Vulva from anterior end	—	4·1–4·8

Kreis gave his measurements as follows:

♀	♂
L = 9·839–12·132	L = 7·362–8·810
a = 19·0–21·3	a = 17·7–19·3
b = 3·3–3·9	b = 3·2–3·4
c = 22·1–22·3	c = 25·6
vu = 48·6–51·5%	

ve	*li*	*oea*	*poe*	*oehi*	ov_1	*vu*	ov_2	*an*	
—	0·45	2·1	10·7	27·9	36·7	50·1	87·7	95·5	= 10·986
0·7	1·1	1·7	3·4	5·3	8·0	6·0	3·4	2·0	
					te				
—	0·52	2·2	15·1	30·3	44·4	*M*		95·6	= 8·086
0·8	1·2	1·6	3·4	4·1	4·2	6·6		2·3	

ve = Head. *li* = Base of Lips. *oea* = Pharynx. *poe* = Excretory pore. *oehi* = Posterior end of oesophagus. *ov* = Ovary. *vu* = Vulva. *an* = Anus. *te* = Testis. *M* = Centre of body.

Spinicauda sp. Baylis & Daubney, 1923

Baylis & Daubney (1923) record a few immature specimens from the duodenum of (?) *Lyriocephalus scutatus* under the name *Spinicauda* sp. and state that they are unable to determine the species. It is very probable that these actually represent a *Meteterakis* sp., probably *M. cophotis* of which *L. scutatus* is a known host.

Conclusion

The genus *Meteterakis* has been reconstituted as a distinct morphological and geographical group. The movement of species to it has altered the constitution of the genera *Africana* and *Spinicauda* and I shall now give a list of the species left in these two genera.

Genus *Africana* Travassos, 1920

Africana africana (Gendre, 1909), Travassos, 1920
A. acuticeps (Gedoelst, 1916), Travassos, 1920
A. astylosterni Sandground, 1933
A. brodeni (Gedoelst, 1916), Travassos, 1920

Genus *Spinicauda* Travassos, 1920

Spinicauda spinicauda (Rudolphi, 1819), Travassos, 1920
S. amarali Pereira, 1935
S. australiensis Baylis, 1930
S. campanula (von Linstow, 1879), Travassos, 1920
S. icosiensis (Seurat, 1917), Travassos, 1920
S. mathevossianae Skarbilovitch, 1950
S. sonsinoi (von Linstow, 1894), Travassos, 1920

SUMMARY

1. The genus *Meteterakis* is reconstituted with eight species: *M. govindi*, *M. louisi*, *M. baylisi*, *M. longispiculata*, *M. cophotis*, *M. japonica*, *M. mabuyi* and *M. triaculeata*.

2. *M. cophotis*, *M. japonica* and *M. longispiculata* were previously placed in the genus *Spinicauda*.

3. *M. mabuyi* was previously in the genus *Africana*.

4. *M. triaculeata* was previously in the genus *Ganguleterakis*.

5. *M. baylisi* and *M. louisi* are new species.

6. *Spinicauda bufonis* and, probably, *Africana varani* are considered synonyms of *M. govindi*.

7. *Africana howardi* is considered a synonym of *M. japonica*.

8. A list of the remaining species in the genera *Africana* and *Spinicauda* is given.

I wish to express my thanks to Dr L. Forcart, Naturhistorisches Museum, Basel, the late Dr S. L. Hora, Zoological Survey of India, Calcutta, and Professor J. J. C. Buckley, London School of Hygiene and Tropical Medicine, for allowing

me to examine specimens from their collections, to Miss A. G. C. Grandison, British Museum (Natural History), for checking the host names, to Dr W. J. Rees of the same institute for help and advice during the preparation of this paper, and to Dr H. W. Parker, also of the same institution, for reading it prior to publication.

REFERENCES

BAYLIS, H. A. (1929). Some new parasitic nematodes and cestodes from Java. *Parasitology*, **21**, 256–65.

BAYLIS, H. A. (1935). Two new parasitic nematodes from Ceylon. *Ann. Mag. nat. Hist.* (10), **16**, 187–92.

BAYLIS, H. A. (1936). *The Fauna of British India, Nematoda, I* (*Ascaroidea and Strongyloidea*), pp. i–xxxvi, 1–408, figs. 1–182. London.

BAYLIS, H. A. & DAUBNEY, R. (1923). A further report on parasitic nematodes in the collection of the Zoological Survey of India. *Rec. Indian Mus.* **25**, 551–38.

CHAKRAVARTY, G. K. (1944). On the nematode worms in the collection of the zoological laboratory, University of Calcutta, Part I. Families Heterakidae and Kathlaniidae. *J. Dep. Sci., Calcutta Univ.* (N.S.) **1**, 70–7.

HSÜ, H. F. (1933). On some nematodes collected in China. *Parasitology*, **24**, 512–41.

INGLIS, W. G. (1957). The comparative anatomy and systematic significance of the head in the Nematode family Heterakidae. *Proc. zool. Soc. Lond.* **128**, 133–43.

KARVE, J. N. (1930). Some parasitic nematodes of frogs and toads. *Ann. trop. Med. Parasit.* **24**, 481–91.

KOO, S. Y. (1939). Nematode parasites of *Bufo melanostrictus*, the common toad from Canton. *Lingnan Sci. J.* **18**, 143–54.

KREIS, H. A. (1933). Ein neuer parasitischer Nematode aus *Corucia zebrata* (Scincidae; Reptilia), *Ganguleterakis triaculeatus* n.sp. *Z. Parasitenk.* **6**, 332–8.

KUNG, C. C. & WU, H. W. (1945). Parasitic nematodes of amphibians from Pehpei, Szechwan, China. *Sinensia*, **16**, 73–83.

LI, H. C. (1933). Report on a collection of parasitic nematodes, mainly from North China. *Chin. med. J.* **47**, 1307–25.

LÓPEZ-NEYRA, C. R. (1947). *Helmintos de los Vertebrados Ibéricos*, II, pp. 413–802. Granada.

MADSEN, H. (1950). Studies on species of *Heterakis* (Nematodes) in birds. *Dan. Rev. Game Biol.* **1**, 1–42.

MAPLESTONE, P. A. (1931). Parasitic nematodes obtained from animals dying in the Calcutta Zoological Gardens. *Rec. Indian Mus.* **33**, 71–171.

MAPLESTONE, P. A. (1932). The genera *Heterakis* and *Pseudaspidodera* in Indian hosts. *Indian J. med. Res.* **20**, 403–420.

SKRJABIN, K. I. (1949). [*Parasitic Nematodes and the Illnesses Caused by Them*, I] (Moscow) (in Russian), pp. 1–613, figs. 1–219.

TRAVASSOS, L. (1920). Esboço de uma chave geral dos Nematodes parasitos. *Rev. Vet. Rio de J.* **10**, 59–70.

WILKIE, J. S. (1930). Some parasitic nematodes from Japanese amphibia. *Ann. Mag. nat. Hist.* (10), **6**, 606–14.

YAMAGUTI, S. (1935). Studies on the helminth fauna of Japan. Part 10. Amphibian nematodes. *Jap. J. Zool.* **6**, 387–92.

YAMAGUTI, S. (1941). Studies on the helminth fauna of Japan. Part 34. Amphibian nematodes, II. *Jap. J. Zool.* **9**, 397–408.

YORKE, W. & MAPLESTONE, P. A. (1926). *The Nematode Parasites of Vertebrates*, pp. i–ix, 1–538, figs. 1–307. London.

APPENDIX

Since this paper was submitted for publication Freitas has published a review of the family Heterakidae (Freitas, J. F. T. (1956), Notas sôbre 'Heterakidae' (Railliet & Henry, 1914 (Nematoda, Subuluroidea), *Rev. bras. Biol.* **16**, 461–82), in which

he suggests many radical changes in the classification of the family, several of which are at variance with the conclusions reached in this paper. Thus, he refers *Africana acuticeps* Gedoelst, 1916, and *A. brodeni* Gedoelst, 1916, to the genus *Meteterakis*, but neither of these nominal species possesses the combination of characters here considered diagnostic of that genus, and I refer them back to the genus *Africana*, which is well founded. In addition, Freitas creates a new genus *Heterakoides* for *Ganguleterakis triaculeatus* Kreis, 1933, and a new genus, *Pareterakis*, for *Africana howardi* Li, 1933. Attention has been drawn above to the differences between the former species and the typical generic pattern of *Meteterakis*, but I still do not feel that they warrant generic recognition, and I here consider *Heterakoides* a synonym of *Meteterakis*. There is no doubt that the latter species, *Africana howardi*, is indistinguishable from *M. japonica* and so I consider *Pareterakis* a synonym of *Meteterakis*. Finally, Freitas mentions a species *Africana singaporensis* Sandosham, 1954, which I had overlooked when preparing this paper and which possesses all the characters diagnostic of *Meteterakis*, as re-defined above, and I take this opportunity of referring it to this genus. *M. singaporensis* appears to be similar to *M. louisi*, but I prefer to leave them distinct until the specimens of the former species have been re-studied and compared with those of the latter.

(*MS. received for publication* 1. XI. 1956.—Ed.)

FURTHER OBSERVATIONS ON THE STRUCTURE OF NEMATODE CUTICLE

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(With Plates VII and VIII)

INTRODUCTION

The cuticles of a large number of nematodes have been examined by Chitwood (1937) who found that they were in all cases composed of several layers. He came to the conclusion that differences were due to thickness and not to structure.

The structure of the cuticle of *Ascaris lumbricoides* var. *suis* has been examined either in whole or in part by several investigators in recent years (Faure-Fremiet & Garrault, 1944; Picken, Pryor & Swann, 1947; Rudall, 1955; Bird & Deutsch, 1957). However, no other adult zooparasitic nematode has been examined recently, and for this reason it was decided to examine the cuticles of *Oxyuris equi* and *Strongylus equinus*, two common parasites of the horse, and to compare their structures with each other and with those of *Ascaris lumbricoides*.

Martini (1912) examined the structure of the cuticle of *Oxyuris equi*. He described outer and inner cortical layers, the former being subdivided into 'ringlets' and 'little ringlets', a 'fibrillar' layer consisting of two distinct layers of fine fibres similar to the fibre layers but much finer, a homogeneous layer and two fibre layers. He was unable to identify a boundary layer or basal lamella and was also unable to see any trace of 'Saftbahnen' (Toldt, 1899).

Chitwood (1937) found that the cuticle of *Strongylus equinus* resembled that of *Ascaris lumbricoides* in many ways: it had external and internal cortical layers, a 'fibrillar' layer, a homogeneous layer, a boundary layer, two fibre layers and a basal lamella.

MATERIAL

Specimens of *Ascaris lumbricoides* var. *suis*, *Strongylus equinus* and *Oxyuris equi* were obtained alive and taken to the laboratory in Ringer's solution (Baldwin & Moyle, 1947) at 38° C.

METHODS

The cuticles were removed as previously described (Bird, 1956).

Surface preparations and sections were obtained and examined under the light and electron microscopes as previously described (Bird & Deutsch, 1957).

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RESULTS

(1) *Oxyuris equi*

(1) *Surface preparations*

An examination of the surface layer, by focusing directly on to the cuticle from above, clearly shows the transverse grooves or furrows which are a great deal further apart in the cuticle of this animal than in *Strongylus equinus* or *Ascaris lumbricoides*.

Layer stripping reveals the presence of the fibre layers (Pl. VII, fig. 1) which can be seen to cross each other at an angle of 120° and to make an angle of 60° with the long axis of the worm.

(2) *Sections*

Longitudinal, transverse and tangential frozen sections, fixed in formal-calcium and mounted in glycerine jelly, have been examined under phase contrast at high power and under oil immersion.

Longitudinal sections under high power (Pl. VII, fig. 2) clearly show, in addition to all the structures mentioned by Martini (1912), that in the vicinity of each transverse striation or groove there is a most interesting pair of structures which traverse the homogeneous layer and connect Martini's so-called 'fibrillar' layers with the fibre layer. Under oil immersion it can be seen (Pl. VII, fig. 3) that each of these structures consists of a bundle of several fine strands which behave peculiarly in that the bundle on the left (Pl. VII, fig. 4) appears to penetrate the cortical layer and to connect with irregularities on the floor of each transverse groove (which Martini has named 'little ringlets'), whilst the bundle on the right does not penetrate the cortex but turns right and runs between it and Martini's 'fibrillar' layer with which it appears to connect before crossing to the next groove.

Tangential sections viewed under high power (Pl. VII, fig. 5) show that Martini's 'fibrillar' layers resemble the fibre layers in that they consist of two layers crossing at an angle to each other. Martini found these to be exactly similar except that the fibres in the 'fibrillar' layer were finer.

In transverse sections under high power (Pl. VII, fig. 6) and under oil immersion (Pl. VII, fig. 7) it can be seen that the 'fibrillar' layers are much thinner, but, as they bear no resemblance to the so-called 'fibrillar' layers of Ascaris cuticle, I have decided to re-name them the outer fibre layers to distinguish them from the two larger, inner fibre layers.

These transverse sections show a layer which has not been mentioned by Martini. It occurs under the inner cortical layer and is about a third as thick. As it appears, in longitudinal sections, to correspond with the previously mentioned connecting strands which branch to the right, I have decided to call this layer and the connecting strands the 'fibrillar' layer.

The cuticle of *Oxyuris equi* thus consists, from the outermost layer inwards, of the following: (1) external cortical layer; (2) internal cortical layer; (3) 'fibrillar' layer; (4) part of homogeneous layer; (5) and (6) two outer fibre layers; (7) and

(8) two inner fibre layers. The homogeneous layer surrounds the outer fibre layers but the greater part lies between them and the inner fibre layers. There is no indication of the presence of a boundary layer or a basal lamella.

(2) *Strongylus equinus*

(1) *Surface preparations*

Examination, from above, of the surface layer shows that the transverse grooves or furrows of this worm are very close together.

Electron micrographs of this region (Pl. VII, figs. 8, 9) show that the grooves appear to contain a line of dark objects which probably consist of the tops of the structures arising from the 'fibrillar' layer. The area between the grooves consists of irregular striations which tend to run at right angles to the grooves.

Layer stripping reveals the presence of the fibre layers (Pl. VIII, fig. 10) which can be seen to cross each other at an angle of about 135°.

(2) *Sections*

Longitudinal and transverse formal-calcium fixed frozen sections mounted in glycerine jelly have been examined under phase contrast at high power and under oil immersion. Wax sections, stained with Mallory's triple stain have also been examined.

The cuticle of *Strongylus equinus* is much thicker than that of *Oxyuris equi* and is also thicker than that of *Ascaris lumbricoides*. The thickness of cuticles varies in different worms of the same species but the cuticle of *A. lumbricoides* is about four-fifths as thick as that of *Strongylus equinus* or about 80 μ as compared with 100 μ.

Longitudinal sections under high power (Pl. VIII, fig. 11) show much the same layered structure as that described by Chitwood (1937) but the structures which he terms 'transverse bands of condensation in the cortical layer' are much closer together than he has depicted. These structures, which for the sake of conformity in nomenclature with *Ascaris lumbricoides* (Bird & Deutsch, 1957) are here called 'structures arising from the "fibrillar" layer', appear to be hollow and, when viewed under oil immersion (Pl. VIII, fig. 12), can be seen to run directly under the grooves in the external cortical layer. As they approach the 'fibrillar' layer they broaden out and stop, but vertical extensions continue on into the homogeneous layer. These extensions are only clearly visible under phase contrast. The two large fibre layers are clearly seen in longitudinal section (Pl. VIII, fig. 11) and their structure does not appear to be as fine as depicted by Chitwood. The boundary layer is also visible in longitudinal section, but both it and the basal lamella are easier to see in transverse sections under high power (Pl. VIII, fig. 13). These sections have been cut between the grooves and only show the broader top parts of the 'fibrillar' structures and their thickened roots which constitute the 'fibrillar' layer itself.

The cuticle of *Strongylus equinus* thus consists, from the outermost layer inwards, of the following: (1) external cortical layer; (2) internal cortical layer; (3) 'fibrillar'

layer with its structures passing from the homogeneous layer to under the grooves of the external cortical layer; (4) homogeneous layer; (5) boundary layer; (6) outer fibre layer; (7) inner fibre layer; (8) basal lamella.

(3) *Ascaris lumbricoides*

(1) *Surface preparations*

Surface views of the cuticle after peptic hydrolysis (Bird, 1955) (Pl. VIII, fig. 14) under high power clearly show the circular hollow structures arising from the 'fibrillar' layer. This is probably because their contents have been digested away.

(2) *Sections*

Interesting structures have been observed in transverse sections in the lateral line region (Pl. VIII, fig. 15). These traverse the homogeneous layer and appear to connect the 'fibrillar' layer with the boundary layer. These structures seem to be too regular to be caused by disorientation due to sectioning. The only explanation which I can give for them is that they indicate that, at times, in some regions of the cuticle, there is a close association between the 'fibrillar' layer and the fibre layers as is seen in *Oxyuris equi*.

Transverse sections along the middle of a groove in the lateral line region (Pl. VIII, fig. 16) show that the boundary layer becomes pyramidal and the fibre layers are displaced. The peak of this pyramid appears to have a connexion with the 'fibrillar' layer. This pyramidal structure seems to have been observed by Bastian (1866) and was described more clearly by van Bömmel (1895).

DISCUSSION

The 'fibrillar' layer of *Ascaris lumbricoides* consists of distinct branches which join to form structures which, it has been suggested (Bird & Deutsch, 1957), might appropriately be termed pore canals. They may conveniently be compared with similar structures arising from the 'fibrillar' layer in *Strongylus equinus*. Their hollow structure has been clearly demonstrated in *Ascaris lumbricoides* after peptic digestion. This suggests that the substance which occupies these pore canals is rich in aromatic amino-acids because the specificity of pepsin is adapted to the hydrolysis of peptide linkages involving aromatic amino-acids (Baker, 1951).

It has been shown (Bird, 1957) that the external cortical layer of *A. lumbricoides* is a tanned protein and contains polyphenol oxidase, and it seems logical to assume that the phenols involved in the tanning reaction are brought to the external cortical layer through the pore canals.

These structures are not found in the relatively thin cuticle of *Oxyuris equi*, and it is possible that they may only have a function in cuticles in which the external surface is a considerable distance from the hypodermis or subcuticle.

As has been mentioned (Bird & Deutsch, 1957), it is difficult to obtain either transverse sections in the lateral line region along the middle of a groove or longitudinal sections along the centre of the lateral line. Transverse sections do, however, show that the boundary layer becomes pyramidal in this region and the fibre layers are displaced (Pl. VIII, fig. 16). As has been mentioned, the peak of this

pyramid appears to have a connexion with the 'fibrillar' layer or roots of the pore canals. This connexion resembles, in some ways, Toldt's (1899) 'Saftbahnen'. However, this structure is localized in the region of the lateral line, whereas the 'Saftbahnen' were thought to be present throughout the cuticle as can be seen in Toldt's (1899) drawings of transverse sections. It is probable that a very thin longitudinal section cut in the centre of the lateral line region would show many similar 'Saftbahnen-like' structures. Sections showing structures like this have not been cut during this investigation. However, as has been mentioned, some unfixed frozen transverse sections, cut in the region of the lateral line (Pl. VIII, fig. 15) have shown surprisingly regular fibre-like structures which originate at the boundary layer and run vertically as far as the 'fibrillar' layer. These structures have not been observed in fixed sections and might be caused by displacement of the fibre layers during the sectioning of fresh material, but their size and regularity does not make this seem feasible. These structures could not have been observed by Toldt as they bear no resemblance to his drawings of 'Saftbahnen'. They resemble, in some respects, the structures traversing the homogeneous layer in *O. equi* (Pl. VII, fig. 3). However, transverse sections cut in the middle of one of the transverse grooves have not been obtained in *O. equi* and so it is difficult to make a comparison. Nevertheless, it is interesting to note that these connexions exist, even though they only appear to be transitory in *Ascaris lumbricoides* and have not been observed in *Strongylus equinus*, although this has not been investigated as thoroughly as *Ascaris lumbricoides*. Could it be that in the more primitive oxyurids they play a constant role in the maintenance and secretion of the cuticle, whereas in the ascarids and possibly also the strongyles, where the pore canals presumably have this function, they occur for transitory periods during very active cuticular secretion as, for instance, during moulting or after injury? In order to answer this in a satisfactory manner it will be necessary to examine nematodes during moulting or after artificial injury.

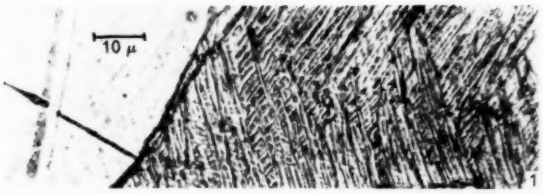
SUMMARY

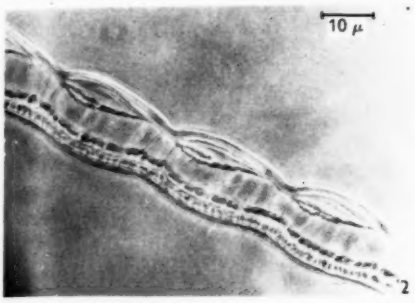
1. An examination of the cuticle of *Oxyuris equi* has revealed the presence of previously unidentified structures. The layers of the cuticle of this nematode have been renamed.

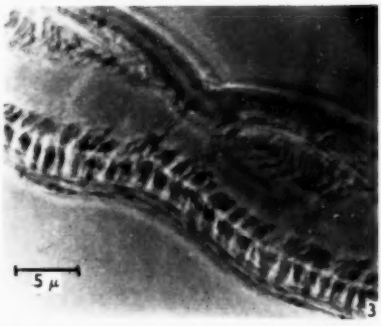
2. The cuticle of *Strongylus equinus* has been examined in a similar fashion and, whilst the basic nomenclature of the various layers has been retained, previous descriptions of the individual layers have been modified and extended.

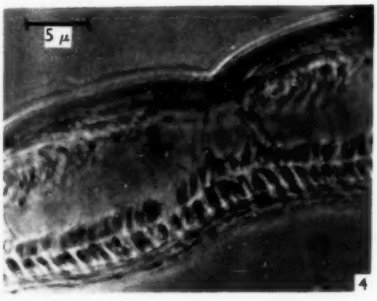
3. Further evidence is put forward in favour of the presence of pore canals in the cuticle of *Ascaris lumbricoides*.

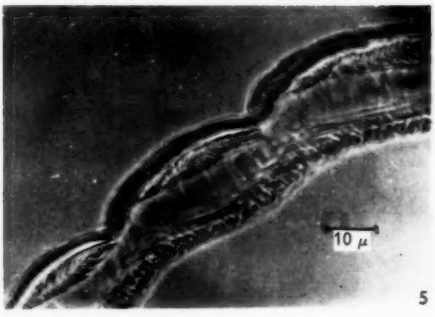
I wish to thank Professor M. M. Swann and Dr C. Rayski of this Department for their advice and criticism, Dr K. Deutsch for the electron microscopy and Mr R. A. Fox, Chief Technician, for making the photographic prints. I should also like to thank the veterinary authorities and meat inspectors at the slaughterhouse, Gorgie, and the Conservator of Animal Health at the Edinburgh Zoological Gardens for their co-operation.

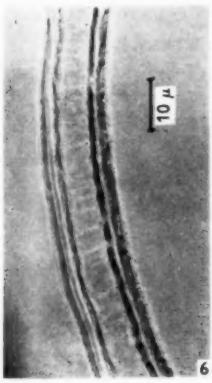


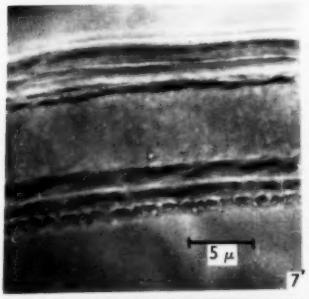


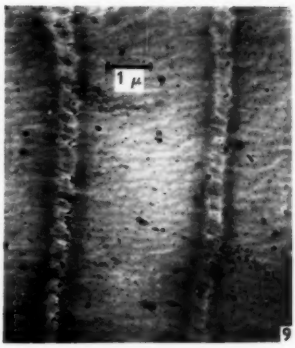












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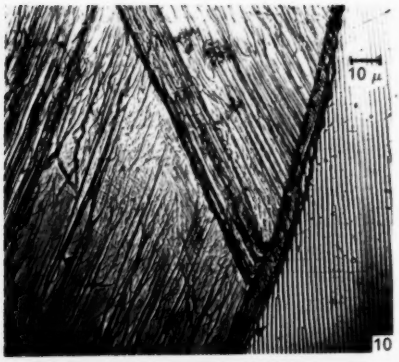
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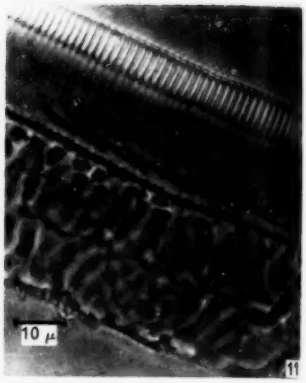
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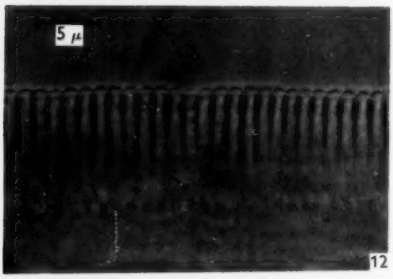
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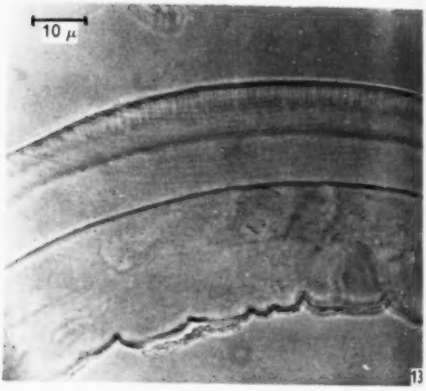
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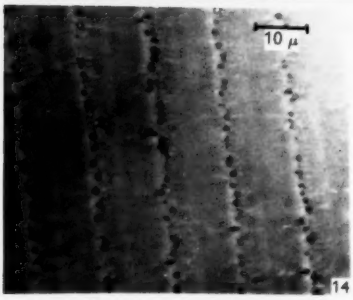
10 μ
14

10 μ
16

10 μ
15

REFERENCES

BAKER, L. E. (1951). New synthetic substrates for pepsin. *J. Biol. Chem.* **193**, 809.

BALDWIN, E. & MOYLE, V. (1947). An isolated nerve-muscle preparation from *Ascaris lumbricoides*. *J. Exp. Biol.* **23**, 277–91.

BASTIAN, H. C. (1866). On the anatomy and physiology of the nematoids, parasitic and free; with observations on their zoological position and affinities to the echinoderms. *Phil. Trans.* **156**, 545–638.

BIRD, A. F. (1957). Chemical composition of the nematode cuticle. Observations on individual layers and extracts from these layers in *Ascaris lumbricoides* cuticle. *Expt. Parasit.* **6**, 383–403.

BIRD, A. F. & DEUTSCH, K. (1957). The structure of the cuticle of *Ascaris lumbricoides* var. *suis*. *Parasitology*, **47**, 319–28.

BIRD, A. F. (1956). Chemical composition of the nematode cuticle. Observations of the whole cuticle. *Expt. Parasit.* **5**, 350–8.

BIRD, A. F. (1955). Importance of proteases as factors involved in the exsheathing mechanism of infective nematode larvae of sheep. *Science*, **121**, 107.

BÖMMEL, A. VAN (1895). Über Cuticular-Bildungen bei einigen Nematoden. *Arb. Würzburg Zool. Institut*, **10**, 191–212.

CHITWOOD, B. G. & M. B. (1937). *An Introduction to Nematology*, Pt. I, pp. 30–2. Baltimore.

FAURÉ-FREMIET, E. & GARRAULT, H. (1944). Propriétés physiques de l'Ascarocollagène. *Bull. Biol.* **78**, 206–14.

MARTINI, E. (1912). Bemerkungen über den Bau der Oxyuren. *Zool. Anz.* **39**, 49–53.

PICKEN, L. E. A., PRYOR, M. G. M. & SWANN, M. M. (1947). Orientation of fibrils in natural membranes. *Nature, Lond.*, **159**, 434.

RUDALL, K. M. (1955). The distribution of collagen and chitin. *Symp. Exp. Biol.* **9**, 49–71.

TOLDT, K. (1899). Über den feineren Bau der Cuticula von *Ascaris megalocephala* Cloquet. *Arb. Zool. Inst. Wien*, **11**, 289–326.

EXPLANATION OF PLATES

PLATE VII

Fig. 1. Surface view of *Oxyuris equi* cuticle showing fibre layers and a transverse groove.

Fig. 2. Longitudinal frozen section, 10 μ thick, of *Oxyuris equi* cuticle formal-calcium fixed, in glycerine jelly, high power, phase contrast.

Figs. 3, 4. Longitudinal frozen sections, 10 μ thick, of *O. equi* cuticle formal-calcium fixed, in glycerine jelly, oil immersion, phase contrast.

Fig. 5. Tangential frozen section, 10 μ thick of *O. equi* cuticle formal-calcium fixed, in glycerine jelly, high power phase contrast.

Fig. 6. Transverse frozen section, 10 μ thick of *O. equi* cuticle formal-calcium fixed, in glycerine jelly, high power, phase contrast.

Fig. 7. Transverse frozen section, 10 μ thick of *O. equi* cuticle formal-calcium fixed, in glycerine jelly, oil immersion, phase contrast.

Figs. 8, 9. Electron micrographs of surface preparations of *Strongylus equinus* cuticle showing the transverse grooves.

PLATE VIII

Fig. 10. Surface view after layer stripping of *S. equinus* cuticle.

Fig. 11. Longitudinal formal-calcium fixed frozen section, 10 μ thick, of *S. equinus* cuticle in glycerine jelly, high power, phase contrast.

Fig. 12. Longitudinal formal-calcium fixed frozen section, 10 μ thick, of *S. equinus* cuticle in glycerine jelly, oil immersion, phase contrast.

Fig. 13. Transverse formal-calcium fixed frozen section, 10 μ thick, of *S. equinus* cuticle in glycerine jelly, high power, phase contrast.

Fig. 14. Surface view of *Ascaris lumbricoides* cuticle after peptic hydrolysis, high power, phase contrast.

Fig. 15. Transverse fresh, unfixed frozen section of *A. lumbricoides* cuticle in the region of the lateral line, 10 μ thick, high power, phase contrast.

Fig. 16. Transverse formal-calcium fixed section in the region of the lateral line, low power, phase contrast.

(*MS. received for publication* 23. XI. 1956.—Ed.)

NEW SPECIES OF *IXODES* TICKS FROM EASTERN AFRICA, WITH A DESCRIPTION OF THE MALE AND NYMPH OF *IXODES OLDI* NUTTALL, 1913

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Department of Zoology, King's College, University of London

(With 91 Figures in the Text)

At the request of Mr Harry Hoogstraal, Head of the Medical Zoology Department, NAMRU-3, Cairo, Egypt, and Dr Gertrud Theiler, Parasitologist, Onderstepoort Veterinary Research Station, South Africa, a survey of the ticks of the genus *Ixodes* in Africa was commenced. This survey has already shown that the numbers of African species of this genus are grossly underestimated, and in a series of papers (some of which are now in press, see References) the systematic status of the existing species are reviewed and new species described. In the present section, new species from East Africa in particular are described, as well as additional data on the female of *I. oldi* Nuttall, 1913, and a description of the hitherto unknown male and nymphs.

The work was commenced when the writer held a Leverhulme Research Award and was continued at the laboratories of the East African Veterinary Research Organization, Muguga, under a Colonial Welfare and Development grant from Her Majesty's Colonial Office. The writer wishes to acknowledge his thanks to these organizations for their financial aid. In addition, he wishes to thank Mr Harry Hoogstraal, Dr Gertrud Theiler, Mr Glen M. Kohls and Miss Jane Walker for their kind assistance and for profitable discussion on various points; also Dr S. E. Piercey, Acting Director of Muguga, for his kindness, and the staff at E.A.V.R.O. for their hospitality. Much of the material described in this paper was received from various institutions, and thanks are due in particular to Dr G. Owen Evans and Mr E. Browning of the British Museum (Natural History) and Dr J. Bequaert, Museum of Comparative Zoology, Cambridge, Mass., U.S.A.

Ixodes rotundatus n.sp. (Figs. 1–6)

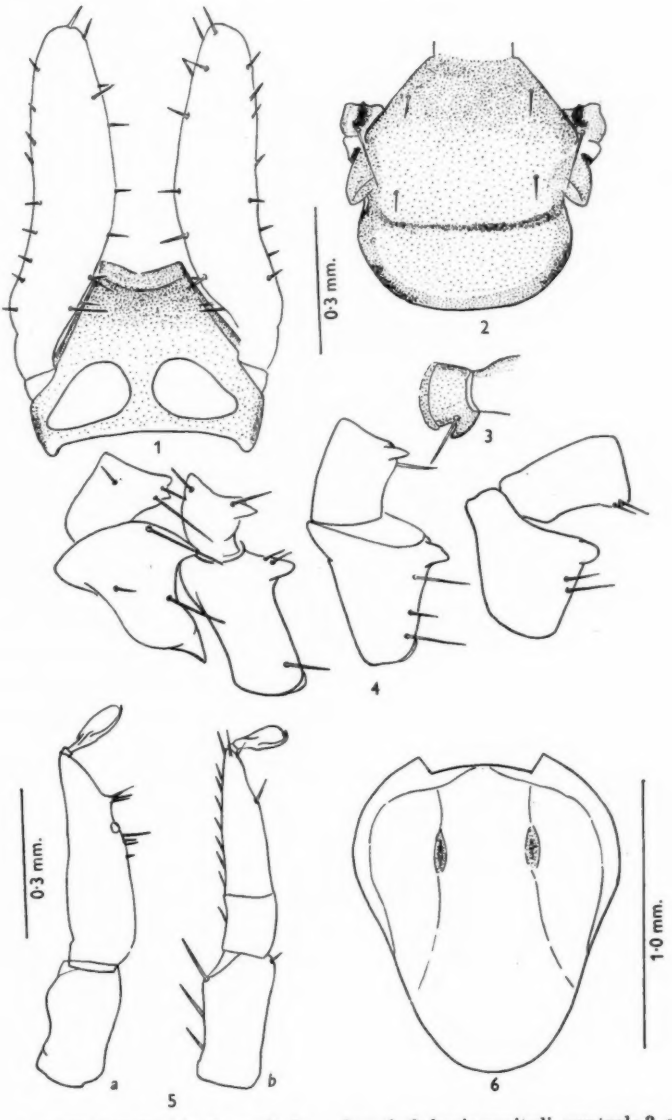
Holotype. Female from a bushbuck, Nakabalide, Kigezi, Uganda, 25.x.1940 (O.P. 2689). Deposited in the Onderstepoort Veterinary Research Station, Onderstepoort.

Paratypes. 3 females with same data as holotype, 1 female from *Tachyoryctes*, Muguga North, Kikuyu. H. Hoogstraal *leg.*, –.vii. 56. Deposited in the Onderstepoort Veterinary Research Station, Onderstepoort and in Hoogstraal's collection, NAMRU-3, Cairo.

Description of female

Five specimens almost circular, measuring 3·7 × 2·9, 3·8 × 3·0, 3·5 × 2·9, 4·1 × 3·0, 3·9 × 2·9 mm. Capitulum and scutum sharply angled to rest of opisthosoma. Sclerotized parts in alcohol-preserved specimens from pale to chestnut brown. Scattered white hairs on opisthosoma and on scutum.

Capitulum (Figs. 1–3). Length from cornua to base of hypostome 0·32 mm., breadth across cornua 0·37 mm., lateral profile lines divergent anteriorly, posterolateral angles drawn out into small cornua with rounded apices; posterior margin slightly concave, trenchant and salient. Surface crazed with few scattered fine punctations, generally flattened but curving to rostrum. Porose areas depressed,



Figs. 1–6. *I. rotundatus* female: 1, capitulum, dorsal; 2, basis capituli, ventral; 3, side view of palpal article 1; 4, coxae I–IV; 5*a*, tarsus I and metatarsus I; 5*b*, tarsus IV and metatarsus IV; 6, scutum.

subtriangular or piriform, large, separated by a narrow interval. Palpi broad, four times as long as broad, narrow strongly to base, concave on outer margin, with distinctive baso-lateral protuberance, convex on mesial surface; length of article 2, 0·37 mm., article 3, 0·26 mm. Hairs short laterally, longer mesially, particularly proximally and mesially. Ventrally postero-lateral and posterior margins broadly rounded, distinct transverse groove at level of well-defined waist just behind auriculae, latter broad, tapering, directed postero-ventrally. Surface flat except for slight upward curvature postero-laterally and in front of the palpal insertion. Pair of bristles subtending base of hypostome on each side and bristles of similar size mesial to auriculae (Fig. 2). Palpal article 1 broader than long, backwardly directed keel-like spur mid-ventrally (Figs. 2, 3). Colour chestnut brown, bordered by a broad blackish margin. No specimen has complete hypostome.

Scutum (Fig. 6). Longer than broad, length 1·29 mm., breadth across the 'shoulders' in front 1·05 mm., greatest width well in front of the mid-line; postero-lateral borders slightly curved inwardly, rounded posteriorly. Cervical grooves shallow anteriorly, but leading to a depression as indicated in Fig. 6, shallowing out postero-laterally. Punctations large, shallow, uniformly distributed. Colour yellow or brown, antero-lateral and proximal half of lateral margins more heavily pigmented than the rest of the scutum (indicated by lines in Fig. 6). Hairs few, scattered, fine, white. Lateral carinae ill defined.

Legs. Long, coxae II and III elongate, narrow (Fig. 4), coxa I with large broad external spur, internal spur of similar size directed towards the mid-line, external spurs on coxae II and III large, approximately of similar size, somewhat hook-like, external spur on coxa IV large, lobate; postero-internally coxae II and III bear less heavily sclerotized marginal saliences; all coxae slightly convex, well supplied with long hairs. Trochanter spurs on legs I–III as sharply pointed spurs increasing in size from I to III, smaller and less well-defined spur on trochanter IV. Tarsus I rather broad, particularly near the proximal end, narrows suddenly to the tip beyond Haller's organ without a hump; length of tarsus I, 0·42 mm., metatarsus I, 0·24 mm.; tarsus IV (Fig. 5*b*) considerably narrower than tarsus I, length 0·42 mm., metatarsus IV, 0·26 mm.

Anal groove. Horseshoe-shaped, arms behind the anus closely convergent.

Genital aperture. Level with posterior half of coxae IV; genital grooves widely divergent to the level of anus, thereafter slightly convergent.

Related species. I. rotundatus shares the characters of auriculae and external spurs on all coxae with *I. theilerae* (name amended from *theileri*) Arthur, 1953, *I. daveyi* Nuttall, 1913, *I. euplecti* n.sp., *I. lunatus* Neumann, 1907, *I. schillingsi* Neumann, 1901 and *I. lemuris* n.sp. (Arthur, 1957*a*). From these species *I. rotundatus* is recognizable on the shape of the ventral ovate basis capituli, the pattern of the auriculae and the ventral spur of palpal article 1; in none are the palpi so markedly pedunculate, and the external margin so concave as in this species.

Distribution. Known at present from Uganda, Kenya.

Hosts. Bushbuck probably (information supplied by Dr G. Theiler), *Tachyorhyctes* (the mole rat).

Ixodes auriculaelongae n.sp. (Figs. 7–12)

Holotype. Female, from mouse, Serere no. 9, Morogoro, Tanganyica (author's note, presumably Tanganyika), –. xii. 1931. W. V. Harries. G. H. F. Nuttall collection no. 3906. Deposited in British Museum (Natural History).

Paratype. Female. Same data as holotype, '1932–11–29–1'.

Description of female

Both specimens partially engorged; sclerotized parts brown with scutum and margins of the basis capituli more deeply pigmented than legs and palpi. Length of the gorged holotype specimen 6·0 mm., breadth 3·2 mm., greatest breadth behind the spiracle.

Capitulum (Figs. 7, 8). Length 0·82 mm., greatest width dorsally across the cornua 0·39 mm.; basis capituli subtriangular, lateral margins in front of palpal insertion long, straight, convergent to cheliceral foramen, behind palpal insertion sides either straight or slightly concave, before bulging outwards postero-laterally to form the outer limit of large, broad, rounded cornua, which are upwardly directed, posterior margin straight, salient and overlapping front margin of scutum (shaded area in Fig. 10). Porose areas large, rounded, depressed, paler than rest of basis, inter-porose interval equal to half or less of their greatest diameter. Palpi originate nearer posterior margin of basis capituli; of moderate length, very narrow basally before becoming widest at about mid-length, do not extend as far forward as level of hypostomal tip; outer profile line a little concave, inner profile line convex, being greatest just distal of mid-length, no clearly defined suture line between articles 2 and 3; combined length of articles 2 and 3, 0·64 mm., about five times as long as broad. Hairs few and short laterally, longer mesially and basally. Ventrally, basis capituli long, narrow, roughly pentagonal, posterior margins slightly convex, postero-lateral angles rounded, sides weakly constricted (Fig. 8); auriculae particularly long, very broad basally, taper to a point with a considerable overlap of the anterior portion of coxae I, directed backwards, outwards and downwards, origin of auriculae at about mid-length of basis capituli indicating greatest width of latter. Surface flat, shiny, impunctate. Palpi flattened on mesial surfaces. Hypostome (Fig. 9): length 0·42 mm., tapers gradually to tip, the shape of the latter cannot be ascertained owing to breakage, visible teeth arranged in 4 rows of 2/2 teeth and thereafter 3/3 nearly to the tip; teeth sharply pointed, mesial teeth smaller than laterals. Median ventral triangular, unarmed area.

Scutum (Fig. 10). Anterior margin overlapped by dorsal ridge and cornua of basis capituli and not visible *in situ*. Length 1·15 mm., breadth 0·70 mm., greatest breadth well behind the middle where lateral margins dilated, posteriorly sides somewhat indented before the posterior edge which is broadly curved. Surface shining, reticulately patterned, few long hairs: large moderately deep punctations, uniformly distributed; lateral carinae as prominent straight ridges which *in situ* appear to rise beneath cornua of basis and extend back to the indentation on the postero-lateral edge, short steep slope from carinae to median field, and laterally declivitous slope to the edge; cervical grooves seen as slightly

Figs. 7–12*b*. *I. auriculaelongae* female: 7, capitulum, dorsal; 8, capitulum, ventral with coxae I; 9, hypostome; 10, scutum; 11, coxae II–IV; 12*a*, tarsus and metatarsus I; 12*b*, tarsus and metatarsus IV.

sinuous depressions which fan out at the level of the indentation, being deepest midway along but anteriorly becoming very shallow and difficult to detect. Scapulae very slight pointed prominences, emargination practically negligible. After removal of the basis capituli the anterior margin of coxae I is visible beneath the scapula (broken line to left of scutum in Fig. 10).

Legs. Moderate length, but segments other than coxae are thin; length of tarsus I (Fig. 12*a*), 0·53 mm., metatarsus I, 0·29 mm.; length of tarsus IV (Fig. 12*b*), 0·41 mm., metatarsus IV, 0·34 mm. Coxae (Figs. 8, 11) flattened, very faintly crazed, coxae I–III are syncoxae, coxae I with broad pointed external spur, internal spur very small and separated from trenchant posterior margin by slight notch, coxa II trenchant behind, more or less square, no spurs, coxa III more or less square, devoid of spurs, coxa IV with small lobate postero-lateral extension, otherwise mesial surface rounded, no internal spur.

Spiracular plate. Small (about as large as coxa IV), round, far removed from coxa IV.

Anal groove. Horsehoe-shaped.

Genital aperture. Situated between coxae III and IV.

Related species. It is difficult to associate this with any group of species or a single species in this genus. In a general way it has the *facies* of members of the *I. nairobiensis* complex. It is easily recognizable by its more or less square coxae, the reduced internal spur on coxa I, long pointed auriculae and the elongate scutum with its broadened posterior extremity.

Hosts. 'Mouse'.

Distribution. Known only from these two specimens collected at Morogoro, Tanganyika.

Remarks. These specimens were originally identified as *I. nairobiensis* by Nuttall and Warburton.

Ixodes nchisiensis n.sp. (Figs. 12A–22)

Holotype. Male from *Petrodromus* sp., Nchisi Mountain, Nyasaland, 10. xii. 1948, A. Loveridge *leg.* Deposited in the Museum of Comparative Zoology, Cambridge, Massachusetts.

Allotype. Female, same data as holotype.

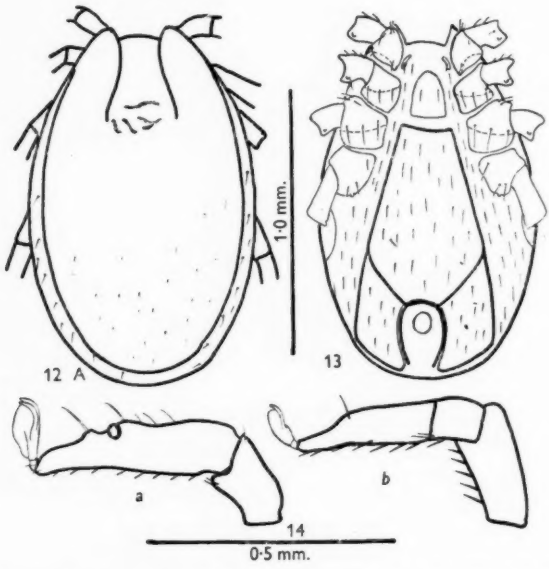
Paratype. 1 female, same data as holotype.

Description of male

Very small, 1·71 mm. long, 0·87 mm., across the spiracles.

Capitulum (Figs. 14A, 15). Overall length 0·29 mm., greatest breadth 0·21 mm., basis capituli pentagonal, posterior margin straight, lateral margins straight and divergent anteriorly to palpal base. Pale brown base colour, periphery more heavily pigmented, surface smooth and convex. Palpi broad, article 2 broadest apically, articles 2 and 3 of approximately equal length, hairs of moderate length laterally, two very long hairs arise meso-basally from article 2. Ventrally (Fig. 15) basis capituli broad, lateral and posterior margins slightly concave, divisible into anterior and posterior regions by transverse ridge extending to palpal bases,

drawn out meso-posteriorly into a pronounced spine, 'auricular' spurs lacking, ridge slopes sharply to base of hypostome from which it is sharply delineated by concave fold; posterior to ridge declivitous to convex surface of posterior half, palpal article 1 drawn out into spur-like projection, bearing moderately long hairs; hairs on other palpal articles short. Hypostome (Fig. 16) short, 0·13 mm., apex rounded, broad, five transverse rows distal to large tooth (basal tooth) which points postero-ventrally, sub-basal tooth short. Dentition of five distal rows from apex to base, 2 of 4/4, 2 of 3/3 (a little asymmetrical) and 1 of $2\frac{1}{2}/2\frac{1}{2}$; with irregular crenulations mesial to large basal and sub-basal teeth.



Figs. 12A–14*b*. *I. nchisiensis* male: 12A, opisthosoma, dorsal; 13, opisthosoma, ventral; 14*a*, tarsus and metatarsus I; 14*b*, tarsus and metatarsus IV.

Scutum (Fig. 12A). Length 1·33 mm., breadth 0·77 mm., widest about mid-length, elongate oval, surface equally convex anteriorly and posteriorly, pale brown colour, finely punctate for the most part, generally smooth except for short shallow irregular depressions at the posterior level of the cervical grooves. Very short hairs most abundant posteriorly. Cervical grooves as short, shallow depressions. Marginal fold narrow, pale yellow, hairs longer than those on scutum.

Venter (Fig. 13). Median and adanal plates longer than broad; surface with few small irregularly distributed punctations: hairs numerous, longer than those of scutum and of about equal length to those on marginal fold.

Legs. Coxae (Fig. 13) I–III having extensive syncoxal areas, coxa I broad, triangular, internal spur small pointed projection, no external spur; spurs lacking on coxae II and III, coxae IV triangular with short, pointed, external spur.

Tarsus I (Fig. 14*a*) somewhat undulate as far as Haller's organ, thereafter tapering rather rapidly without a pronounced hump, hairs on ventral surface of moderate length, these hairs become stronger and longer on legs I–IV; tarsus IV (Fig. 14*b*) tapers to the apex, particularly beyond the telotarsus, hairs long and spinose ventrally; length of tarsus I, 0·42 mm., metatarsus I, 0·19 mm.; tarsus IV, 0·39 mm., metatarsus IV, 0·30 mm. Trochanter spurs short, pointed on legs I–IV. Pulvillus as long as claws.

Spiracular plate. Elongate oval, macula antero-ventral.

Anal groove. Horsehoe-shaped.

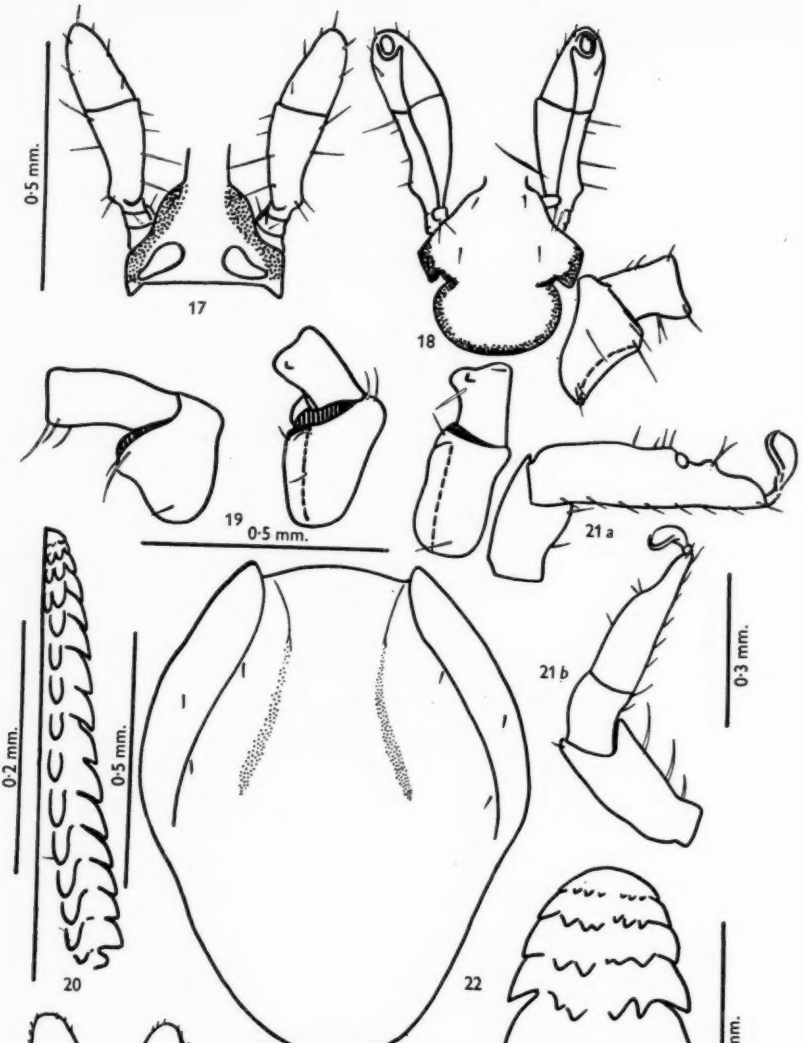
Description of female

Capitulum (Figs. 17, 18). Small, overall length 0·55 mm., breadth 0·32 mm., surface flat, brown, heavily pigmented marginally, posterior margin straight, salient, drawn out into pointed triangular cornua, lateral margins almost straight, parallel, carinate. Porose areas pear-shaped, interporose interval greater than maximum width. Palpi more than thrice the greatest breadth, mesial profile slightly convex, apex of article 2 wider than base of article 3, lateral profile forms prominent bulge at base of article 2, latter narrowing appreciably proximal to this, beyond bulge article 2 concave and widens to the suture line, article 3 tapers to its rounded apex; length of article 2, 0·22 mm., article 3, 0·17 mm. Hairs few, long. Ventrally (Fig. 18), basis capituli broadest across auriculae, latter as truncated straight-edged projections, distinct lateral elevations separate auricular portion of basis capituli from broadly rounded posterior margin (Fig. 18), auriculae and margins most heavily pigmented, two pairs of hairs on front half of basis capituli as figured (Fig. 18); palpal article 1 drawn out into prolongation as in male. Hypostome (Fig. 20): length 0·35 mm., narrows to rounded tip; largest teeth near proximal end; dentition from apex to base, 4 rows of 3/3, 11 rows of 2/2.

Scutum (Fig. 22). Length 0·98 mm., breadth 0·76 mm., broadest about midway along its length, antero-lateral margins gently curved to scapulae; postero-lateral margins more strongly convergent, sinuous, posterior margin narrowly curved. Surface flat between curved, elevated lateral carinae; beyond the latter surface strongly curved; cervical grooves as short, divergent, shallow depressions. Alloscutum well supplied with moderately long white hairs. Punctations small, scattered; hairs few, short, white.

Legs. Moderate length, coxae II and III (Figs. 18, 19) having extensive syncoxal areas; coxa I triangular, with very small tapering postero-internal salience, hairs long, fine; coxae II and III longer than broad, spurs lacking; coxae IV subtriangular, with external spur as a tuberosity, hairs long and sparse. Trochanter spurs small but distinct on legs I–III, but indecisive on trochanter IV. Tarsi (Figs. 21*a*, *b*) similar pattern to those of male, length of tarsus I, 0·48 mm., metatarsus I, 0·25 mm.; length of tarsus IV, 0·48 mm., metatarsus IV, 0·32 mm.

Related species. I. nchisiensis agrees with *I. oldi* Nuttall, 1913 in (1) the shape of the venter of the basis capituli in the females of both are strongly curved posteriorly and strongly constricted about mid-length, (2) the coxae are devoid of spurs (other than a lobate swelling on coxa IV of *I. nchisiensis*) and the postero-



Figs. 14A–22. *I. nchisiensis*. Male: 14A, capitulum, dorsal; 15, capitulum, ventral; 16, hypostome. Female: 17, capitulum, dorsal; 18, capitulum, ventral; 19, coxae II–IV; 20, left half of hypostome; 21*a*, tarsus and metatarsus I; 21*b*, tarsus and metatarsus IV; 22, scutum.

internal angles of coxae I are indicated by slight tapering marginal saliences in the females and minute pointed spurs in comparable situations on the male. The auriculae differ, in that in *I. nchisiensis* they have straight, truncated edges in contrast to the tapering rounded apical form typical of *I. oldi*. In *I. oldi* females and males the scutum is also more heavily punctate. In the males of *I. oldi* the basis capituli is broadest dorsally about mid-length, tapering in front and behind, the auricular lobes are well defined and almost as large as the median lobe.

Host. Known only from *Petrodromus*.

Ixodes oldi *Nuttall*, 1913 (Figs. 23–47)

I. oldi Nuttall (1913), pp. 135–6, fig. 3 (capitulum, scutum, coxae I–IV, anal groove, spiracle, tarsus IV).

Material examined. Total 20 females, 3 males, 17 nymphs. 6 females, 2 males, 3 nymphs, *Genetta* sp., Adiopodomme, Ivory Coast, 13. vii. 1953; 5 females, 1 male, *Civettictis civetta*, Njalla, Sierra Leone, T. S. Jones *leg.*, British Museum (Natural History); 3 females, bush cat, Komatendu, Sierra Leone, West Africa (Imperial Institute of Entomology, no. 604), (British Museum, Natural History); 1 female, labelled as '*I. rasus* Nm, Bismarckburg (Toga), Conradt *leg.* Neumann det. 1899, Berlin Museum', now in Neumann collection, Toulouse Museum; 2 females, *Genetta genetta neumanni*, Limuru, Kenya, Capt. R. Pomfret *leg.* (collection of Miss Jane Walker, E.A.V.R.O.); 3 females, 4 nymphs, *Genetta*, HH 11234, Melula, Mt Meru, Tanganyika, 6000 ft. elevation, 1. viii. 1956, Hoogstraal and Kohls *leg.*; 1 gorged nymph, *Paraxerus*, Muguga North, Kikuyu, Kenya, 7200 ft., 24. vi. 1956. Hoogstraal *leg.*; 1 nymph, *Genetta* (HH 10903), Rift Valley floor, ± 25 miles South-west of Nairobi, 9. vii. 1956, Hoogstraal and Theiler *leg.*; 6 nymphs, *Genetta* (HH 11439), Muguga North, Kikuyu, Kenya, 7200 ft., 26. vii. 1956, Hoogstraal and Kaiser *leg.*; 1 nymph, *Tachyorhyctes*, Muguga North, Kikuyu, Kenya, 30. vi. 1956, J. Walker *leg.*

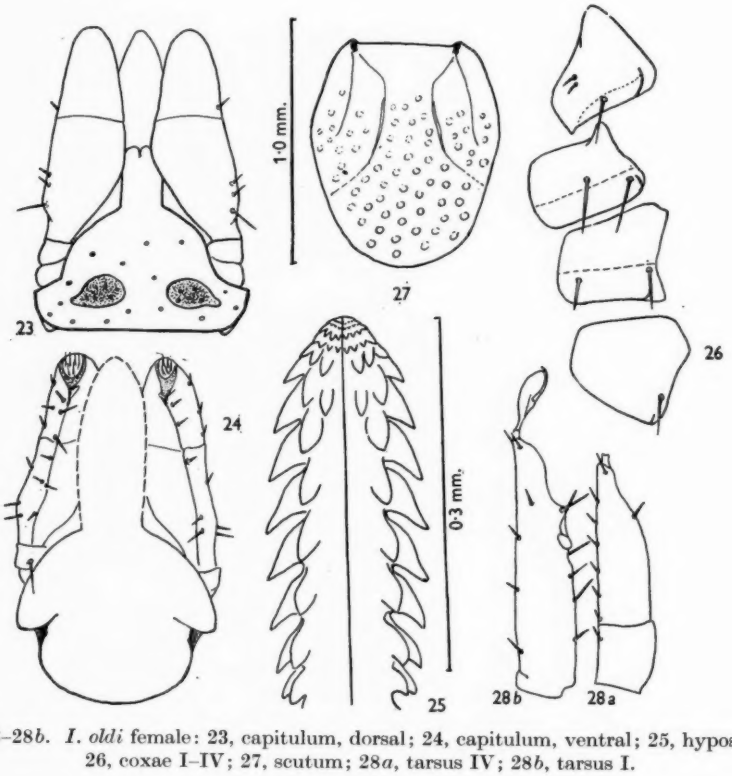
Redescription of female

Holotype female from bush cat, collected at Komatendu, Sierra Leone, West Africa, 13. viii. 1912, J. J. Simpson *leg.*, deposited in British Museum (Natural History).

Body. Sclerotized parts reddish brown in alcohol-preserved specimens, greatest width at level of spiracle, globular when gorged.

Capitulum (Figs. 23–25). Length 0·48–0·50 mm., greatest width of 0·35–0·37 mm. across the basis capituli immediately behind palpal insertion; lateral margins short, strongly divergent anteriorly, carinate, postero-lateral edges rounded, dorsal ridge straight; cornua small. Porose areas small, piriform, depressed, interporose interval about equivalent to their greatest length; dorsal surface flattened, bearing scattered pores, hairless. Palpi two and a half times as long as broad, outer profile either straight or slightly concave, mesial profile particularly convex along the distal two-thirds of article 2; article 3 tapers strongly mesially to the apex, giving a cone-like appearance in dorsal view; hairs moderate in number and short,

excepting a few long ones mesobasally; length of article 2, 0·19–0·21 mm., breadth of article 2, 0·12 mm., length of article 3, 0·14–0·15 mm. Ventrally (Fig. 24), basis capituli constricted at mid-length, broadly rounded behind the auriculae, latter form trenchant rounded tapering protuberances, transverse suture line visible; antero-lateral margins broadly rounded. Surface smooth, shining, impunctate. Hairs on ventral edge of palpus short. Hypostome (Fig. 25): length about 0·32–0·34 mm., denticles as 4 rows of 4/4, 2 rows of 3/3 and then 6 rows of 2/2 to the base, laterals equal in size to medians. Median area unarmed, apex rounded.



Figs. 23–28*b*. *I. oldi* female: 23, capitulum, dorsal; 24, capitulum, ventral; 25, hypostome; 26, coxae I–IV; 27, scutum; 28*a*, tarsus IV; 28*b*, tarsus I.

Scutum. Somewhat variable in shape, some specimens reminiscent of that figured by Nuttall (1913), others of the type indicated in Fig. 27. Length 0·9–1·0 mm., breadth 0·79–0·85 mm. Elongate oval with greatest width at or in front of mid-length, scapulae rounded, small and not as extensive as those figured by Nuttall (loc. cit.), emargination slight; cervical grooves distinct, shallow and just visible to the postero-lateral border; lateral carinae as short ridges arising from near the scapulae but not reaching the lateral border. Punctations many, moderately coarse, fairly uniformly distributed but most prominent between the cervical grooves posteriorly; no hairs.

Legs. Moderate in length and breadth, with long hairs ventrally on tarsi II–IV, tarsus I (Fig. 28*b*) slightly humped, otherwise all taper to their apices; length of tarsus I, 0·53 mm., metatarsus I, 0·27 mm., length of tarsus IV (Fig. 28*a*), 0·46 mm., metatarsus IV, 0·30 mm. Coxae (Fig. 26) flat, coxae I–III with fairly extensive syncoxal areas; smooth, hairs long, posterior margins of coxae I–III trenchant, flanged behind; very small triangular protuberance on postero-internal border of coxa I, trochanters lacking spurs.

Spiracular plate (Fig. 47). Transversely oval.

Vulva (Fig. 28A). Between coxae IV; genital groove rounded anteriorly, diverging at first before converging slightly in front of anus.

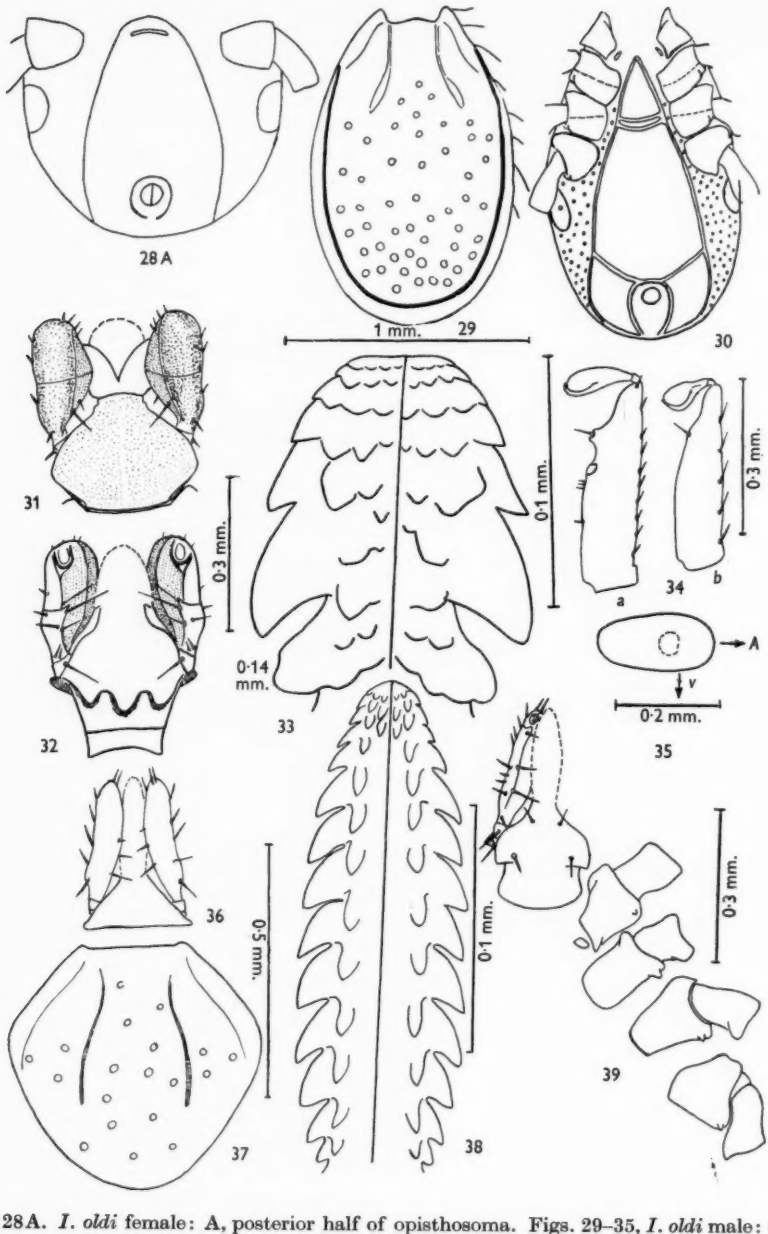
Anal aperture. Located far back on the opisthosoma, anal groove horseshoe-shaped in well-fed specimens, almost a complete ring in unfed or slightly engorged specimens.

Description of male

Allotype. Male, from *Genetta* sp., Adiopodomme, Ivory Coast, 13. vii. 1953. Deposited in British Museum (Natural History). Very small, length 1·6 mm., breadth across the spiracle 0·76 mm.; legs relatively short. Elongate oval.

Capitulum (Figs. 31–33). Length 0·375 mm., breadth 0·27 mm., across the widest portion of the basis capituli at about midway between palpal insertion and posterior border; basis capituli diamond-shaped, slightly broader than long, maximum extension forming flanges which are visible from below (striped in Fig. 32); small but distinct indentation where postero-lateral margin merges with posterior border, lateral and posterior edges salient, posterior margin straight or a little convex, cornua lacking. Surface flat, dark brown colour, reticulate, impunctate. No hairs. Palpi short, about twice as long as broad, articles 2 and 3 of about equal length, combined length 0·23 mm., greatest breadth 0·11 mm. Most of the hairs short, but one or two long hairs mesobasally. Ventrally basis capituli narrows posteriorly, posterior margin extended below the opisthosomatic cuticle. Surface smooth, crazed, impunctate and without hairs; transverse ridge bears auricular lobes and a slightly larger median projection; in front of the ridge, surface slopes strongly to hypostomal base. Palpal articles 2 and 3 flat on mesial surfaces, long hairs arise from the ventral edge of article 2 and a similar hair from lower surface of article 1. Hypostome (Fig. 33): short, length 0·14 mm., broad, tip almost flat, teeth from the apex to the base in seven rows, lateral teeth of distal 6 rows pointed, corresponding median elements as crenulations, 4 distal rows bear 4/4 files, the fifth 3/3 files; sixth row with very large lateral denticle pointing downwards and backwards, mesially two to three incomplete rows of irregular crenulations, proximal lateral tooth irregularly lobed, mesially two rows of irregular crenulations.

Scutum (Fig. 29). Length 1·23 mm., breadth 0·74 mm.; elongate oval in outline, scapulae prominent, rounded at their tips; emargination moderate. Cervical grooves short, ending about one-third the distance of the scutum from the anterior margin, shallow, weak near scapulae, widening posteriorly to form relatively broad depressions; lateral carinae distinct, very short, not attaining lateral margins. Greater part of area between cervical grooves finely punctate, behind and lateral



Figs. 28A. *I. oldi* female: A, posterior half of opisthosoma. Figs. 29–35, *I. oldi* male: opisthosoma, dorsal: 30, opisthosoma, ventral; 31, capitulum, dorsal; 32, capitulum, ventral; 33, hypostome; 34*a*, tarsus I; 34*b*, tarsus IV; 35, spiracle. Figs. 36–39. *I. oldi* nymph: 36, capitulum, dorsal; 37, scutum; 38, hypostome; 39, capitulum and coxae I–IV, ventral view. A, anterior; *v*, ventral.

to these grooves many moderately coarse, uniformly distributed punctations. Lateral body fold broadest between scutum and posterior edge of body; narrows anteriorly and disappears at the level of the second pair of legs.

Ventral plates (Fig. 30). Pregenital plate triangular; median plate nearly twice as long as anal plate, greatest breadth posteriorly; adanal plates slightly longer than anal plates, with concave inner and convex outer borders; anal plate distinctly horseshoe-shaped, with front margin somewhat flattened. Punctations few and of moderate size. The integument lateral to these plates studded with small chitinous hair-bearing placques.

Legs (Figs. 30, 34*a*, *b*). Short, all coxae trenchant, flanged behind, lie close against one another so that the posterior margin of coxae II overlaps the anterior margin of coxae II, and III that of IV; very short internal spur on coxa I. Tarsus I weakly humped, relatively broad, short, the hump decreasing in intensity from tarsi II to IV, length of tarsus I, 0·42 mm., metatarsus I, 0·19 mm., length of tarsus IV, 0·37 mm., metatarsus IV, 0·22 mm.

Spiracular plate (Fig. 35). Elongate oval, long axis parallel to that of body, macula antero-mesially located, length 0·23 mm., breadth 0·12 mm.

Genital opening. On a level with coxae III (Fig. 30).

Description of nymph

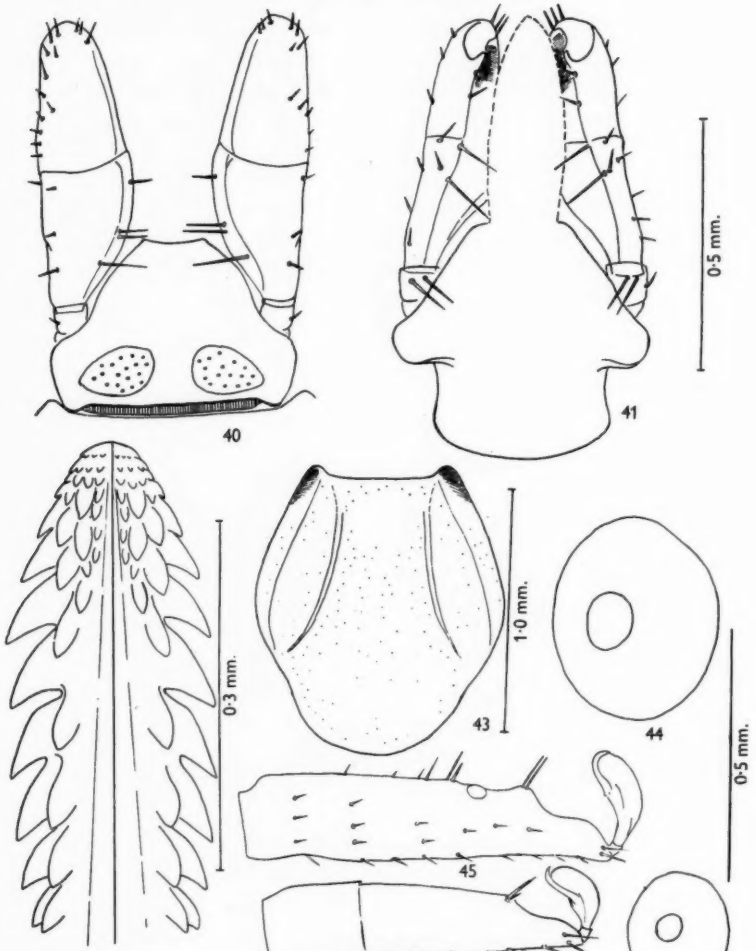
Capitulum (Figs. 36, 38, 39). Length 0·31 mm., breadth 0·20 mm. Basis capituli wide and convex, with lateral margins converging anteriorly. Surface smooth, impunctate. Palpi long, broad, laterally straight or slightly concave, mesially convex, combined length of articles 2 and 3, 0·27 mm., long hairs. Ventrally basis capituli about as long as broad with posterior margin rounded and salient, sides mildly constricted just behind the relatively large spur-like auriculae which are slightly elevated above the general surface, a long hair mesial of each auricula, and another at the base of the hypostome (Fig. 39). Inner faces of palpi flattened, ventral edge with long hairs extending across beneath the hypostome. Hypostome (Fig. 39): long, curved profiles, rounded tip, denticles from apex to base as 3 rows of 3/3 files and 10 rows of 2/2 files to the base, corona lacking.

Scutum (Fig. 37). Length 0·49 mm., breadth 0·51 mm., broadest slightly in front of middle; lateral margin straight in front, postero-laterally curves to a broadly rounded extremity. Punctations few, larger and more numerous in posterior region. Colour yellow, with few or no hairs; cervical grooves extend back to the posterior third, where they broaden; lateral carinae evident as slight elevations in anterior half.

Coxae (Fig. 39). Flattened, coxae I–IV with tapering external spurs; a larger more strongly pointed internal spur on coxa I.

Variants. Material examined: two partially gorged female specimens, *Atilax paludinosus robustus*, Kampala, Uganda, –. ix. 41. (Onderstepoort Research Station). Mean measurements of two specimens: length of capitulum from dorsal ridge to apex of hypostome 0·68 mm., breadth of basis capituli across the cornua 0·45 mm.; length of palpal article 2, 0·26 mm., length of article 3, 0·24 mm.; length of tarsus I, 0·73 mm., length of metatarsus I, 0·40 mm.; length of tarsus IV,

0·63 mm., length of metatarsus IV, 0·47 mm.; length of spiracle (Fig. 44), 0·4 mm.; breadth of spiracle, 0·34 mm.; length of scutum (Fig. 43), 1·18 mm., breadth of scutum, i.e. at its greatest width about midway along, 1·06 mm.



Figs. 40–47. *I. oldi* variant, female: 40, capitulum, dorsal; 41, capitulum, ventral; 42, hypostome; 43, scutum; 44, spiracle; 45, tarsus I; 46, tarsus IV; 47, spiracle of typical specimen of *I. oldi*.

These female specimens differ from typical *I. oldi* female specimens in the arrangement and disposition of the hypostomal teeth, more particularly towards the apex (compare Figs. 25, 42); such dentition differences, particularly near the apex of the hypostome, occur as a normal intraspecific variation in some species

(e.g. *I. cavipalpus*) and the same is true also for the auricular pattern. The legs of the Uganda specimens are very much longer and thicker, as seen from the following mean measurements for legs I and IV:

		Specimens from Ivory Coast, Sierra Leone (mm.)	Specimens from Uganda (mm.)
Leg I:	Tarsus	0·52	0·73
	Metatarsus	0·27	0·40
	Tibia	0·30	0·47
	Femur	0·30	0·47
	Trochanter	0·18	0·26
Total length		1·57	2·33
Leg IV:	Tarsus	0·45	0·63
	Metatarsus	0·29	0·47
	Tibia	0·34	0·56
	Femur	0·47	0·63
	Trochanter	0·23	0·37
Total length		1·78	2·66

The scuta differ in shape and size, but in view of the possibility of intraspecific variations and the already recognized variations in scutal form of typical *I. oldi* females this has little significance for separation. The scutal punctations are however quite distinct; in the female from Uganda the punctations are fine, widely separated and occupy the whole surface except for a small area anteriorly between the cervical grooves; in specimens from the Ivory Coast and Sierra Leone the punctations are coarser and deeper, closer together and more concentrated in the posterior region, anteriorly they are absent or very few. I am hesitant to elevate the two Uganda specimens to varietal rank without knowledge of forms from intervening geographical areas.

Related species. See remarks under *I. nchisiensis*.

Hosts. Genetta sp., *Genetta genetta neumanni*, *Civettictis civetta*, *Paraxerus*, *Helogale*, *Tachyorhyctes*.

Ixodes latus n.sp. (Figs. 48–53*b*)

Holotype. Female from *Sciurus lucifer*, Matipa Wilindi Range, Misuku Mts., Nyasaland, 2 Oct. 1948, A. Loveridge *leg.* Deposited in the Museum of Comparative Zoology, Cambridge, Mass., U.S.A.

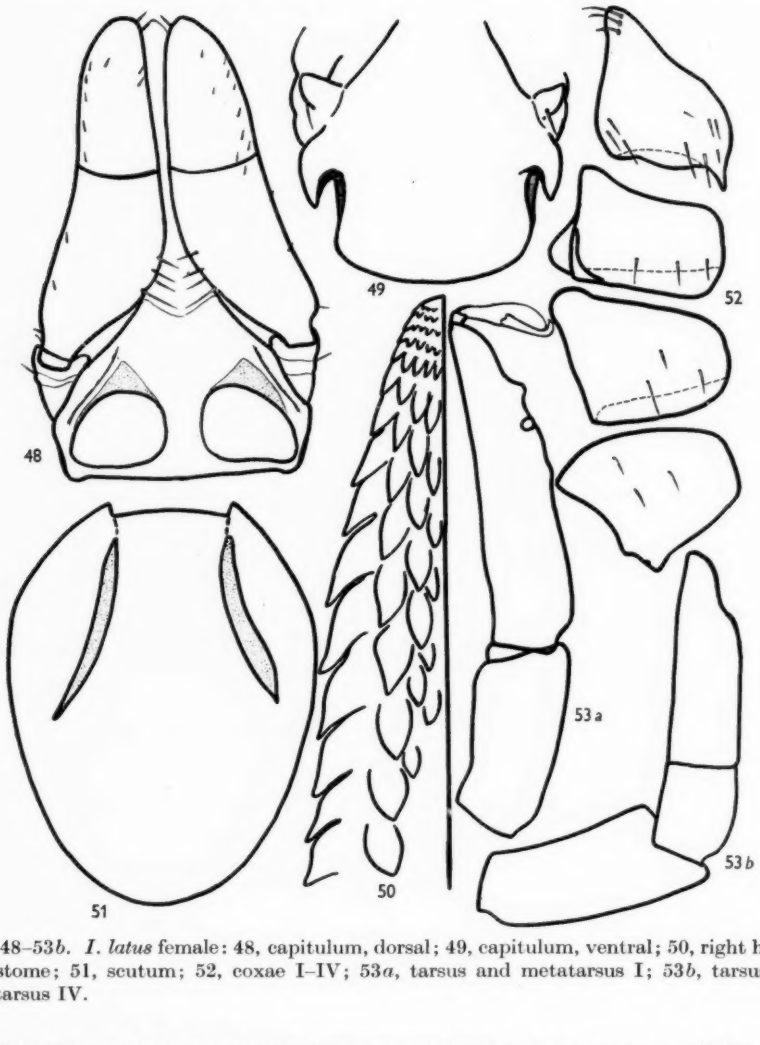
Paratypes. 2 females with same host and locality data as holotype; 1 female Matipa Wilindi Range, Misuku Mts., Nyasaland, 1948. No other data. Deposited in the Museum of Comparative Zoology, Cambridge, Mass., U.S.A.

Description of female

In alcohol-preserved specimens, sclerotized parts dark brown, alloscutum yellow.

Capitulum (Figs. 48, 49, 50). Large, overall length of capitulum 0·91 mm., breadth across dorsal ridge 0·46 mm., posterior margin slightly curved, cornua

very small blunt rounded protuberances, almost straight lateral margins divergent to palpal base, sides of basis capituli steep, pronounced ridge internal to palpal insertion, surface slopes distally. Porose areas large, broader than long, depressed with anterior triangular depression (stippled in Fig. 48) extending forwards, interporose interval narrow. Palpi three and a half times as long as broad, limited



Figs. 48–53*b*. *I. latus* female: 48, capitulum, dorsal; 49, capitulum, ventral; 50, right half of hypostome; 51, scutum; 52, coxae I–IV; 53*a*, tarsus and metatarsus I; 53*b*, tarsus and metatarsus IV.

mesially by broad flange, lateral margins of articles 2 and 3 concave, mesial margins of article 2 broadly convex particularly about mid-length before narrowing appreciably to junction with article 1, article 3 mesial margin straight, tapering to apex, more broadly rounded laterally and more sharply curved mesially. Hairs short and rather inconspicuous apart from longer dorsal meso-basal components.

Ventrally (Fig. 49) basis capituli about as broad as long, narrowed slightly at 'waist', postero-lateral and posterior border broadly rounded, auriculae short, broad, retrograde, heavily pigmented spurs; surface gently curved; ventral periphery heavily pigmented. Hypostome (Fig. 50) narrowing from the base to rounded apex, length 0·44 mm., dentition from apex to base, after a short corona, as 3 rows 5/5 files, 6 rows 4/4 files, 1 row 3/3 files, 2–3 rows 2/2 files.

Scutum (Fig. 51). Length 1·54 mm., breadth 1·15 mm., long oval with tendency to narrow more strongly anteriorly. Cervical grooves narrow and shallow at first, thence leading to broad depressions which again narrow posteriorly, do not attain postero-lateral margin. Lateral carinae very weak, barely visible. Punctations large, shallow, uniform, well spaced, evenly distributed depressions; hairs short, white, shorter than those on alloscutum.

Legs. Long and thick, with long white hairs particularly on ventral surface. Coxa I with broad-based, tapering, pointed, postero-internal spur (Fig. 52), external spur very short, much broader than long, surface slightly convex with hairs of varying length, coxae II and III lacking spurs, coxa IV with small tapering external spur, coxae I–III with narrow syncoxal areas. Tarsi of moderate length and breadth, hairs generally short (Figs. 53*a*, 53*b*), tarsus I slightly humped distally, hump becoming progressively weaker from coxae II to IV, length of tarsus I, 0·68 mm., metatarsus I, 0·41 mm.; length of tarsus IV, 0·61 mm., metatarsus IV, 0·46 mm. Trochanter spurs present.

Genital opening. On a level with coxae IV.

Spiracular plate. Subcircular, macula antero-ventral.

Anal opening. Near posterior border, anal groove horseshoe-shaped, posterior opening more widely divergent in fed specimens.

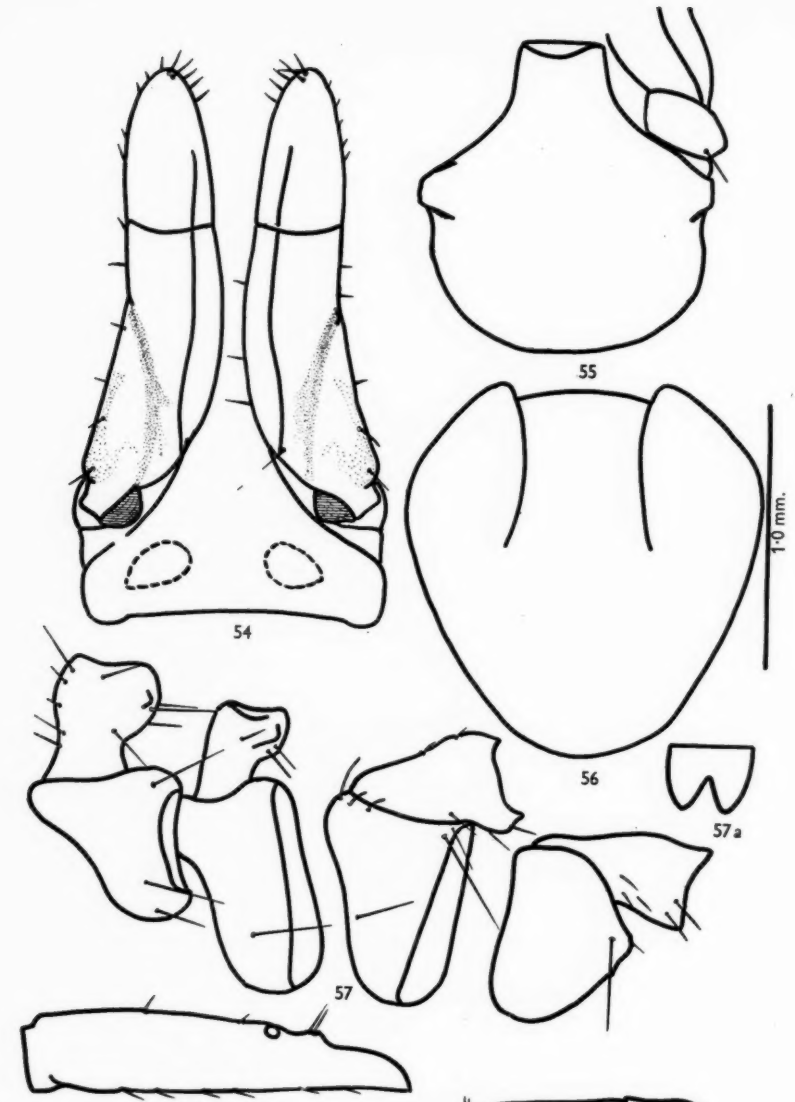
Related species. I. latus has the following diagnostic characters: the cornua are short, indistinct or absent, the auriculae are retrograde spurs, and the external spur on coxa I is much reduced whilst the internal spur on the same coxa is broad basally and tapers to a sharp point. No other known African tick of this genus possesses this combination of characters. The nearest approach is that of *I. lunatus in sensu* of Colas-Belcour and Grenier (1942), but the latter is readily recognized by the more extensive syncoxal areas on coxae I–III, the long tapering external spur on coxae IV and the presence of well-developed cornua (although not noted by the aforementioned writers). (See Arthur (1957*b*) on this problem of *I. lunatus*.)

Hosts. Known only from *Sciurus lucifer*.

Ixodes loveridgei n.sp. (Figs. 54–71)

Holotype. Female, *Cricetomys gambianus viator* Thomas, Mt Cholo, Nyasaland, Arthur Loveridge *leg.*, Deposited in the Museum of Comparative Zoology, Cambridge, Mass., U.S.A.

Allotype. Male, same data as for holotype. Deposited in the Museum of Comparative Zoology, Cambridge, Mass., U.S.A.



Figs. 54–58*b*. *I. loveridgei* female: 54, capitulum, dorsal; 55, capitulum, ventral; 56, scutum; 57, coxae and trochanters I–IV; 57*a*, genital apron; 58*a*, tarsus I; 58*b*, tarsus IV.

Description of female

Large species with prominent capitulum, stout, long legs and an alloscutum with long, fine, curved hairs.

Capitulum (Figs. 54, 55). Basis capituli nearly twice as broad as long, posterior margin straight, salient with broad inconspicuous bulges in position of cornua, postero-lateral angles rounded, lateral margins very short, slightly rounded, becoming more accentuated behind palpal insertion in direction of midline; surface generally flattened except for slight elevations postero-laterally. Porose areas small, piriform, depressed, separated by a distance greater than their maximum length. Extensive intersegmental membrane between basis capituli and article 1. Length from dorsal ridge to palpal extremities 1·09 mm., breadth across basis capituli 0·6 mm. Palpi straw colour, asymmetrically concave with dorsal overlap greater than that of the ventral, long, about $4\frac{1}{4}$ times as long as broad, broad mesial dorsal flange, length of article 2, 0·6 mm., article 3, 0·33 mm. (in Fig. 54 the palps are slightly foreshortened); greatest breadth of 0·21 mm., nearer proximal end of article 2; lateral profile of article 2 straight for proximal three-quarters beyond basal bulge, then sharply angled (Fig. 54); a depression originating at lateral angularity passes backwards and inwards, laterad of the depression is an elevation which is continuous with the proximal swelling; distal to the depression surface gently convex, seen dorsally this gives the palp a twisted appearance; mesial profile as a broad arc, article 3 almost cone-like. Hairs of moderate length except proximally on article 2, and at apex of article 3. Article 1 broader than long, with brown cushion-like expansion mesially which envelops base of article 2 (shaded in Fig. 54) and continued ventrally into a broad spur. Ventrally (Fig. 55) basis capituli very broad, length to breadth as 1·0:0·63, gently rounded posteriorly and postero-laterally, constriction slight, in front of latter short, broad, straight-edged auriculae which are readily recognizable.

Scutum (Fig. 56). Length, 1·4 mm., breadth 1·34 mm., broadest across the anterior third, antero-lateral margins short, postero-lateral margins long, almost rectilinear, posterior margin broadly rounded, lateral carinae lacking; cervical grooves very shallow, short, limited to anterior half; scapulae broad, rounded in front, emargination moderate. Punctations shallow, small, uniformly distributed.

Legs. Long, of moderate thickness, coxae I–III (Fig. 57) with narrow syncoxae, very much longer than broad, gently convex, impunctate with long fine hairs, coxa I subtriangular, with marginal salience on postero-internal edge, no external spur; slight marginal salience externally on coxa IV, otherwise spurs absent on all coxae; short, indistinct postero-internal marginal saliences on coxae I–III. Tarsi (Figs. 58*a*, 58*b*) long, tarsus I narrow, tapering with slight hump distal to Haller's organ, hairs of moderate length, similar hump on tarsus IV, hairs longer and stronger, becoming increasingly spinose from leg I to IV and from distal to proximal segments, length of tarsus I, 0·79 mm., metatarsus I, 0·46 mm., tarsus IV, 0·67 mm., metatarsus IV, 0·52 mm.

Genital opening. Between coxae IV, overlapped by broad, prominently bilobed apron (Fig. 57*a*).

Spiracular plate. Transversely oval, macula central, goblets large.

Anal opening. Located far back, completely surrounded by circular anal grooves.

Description of male

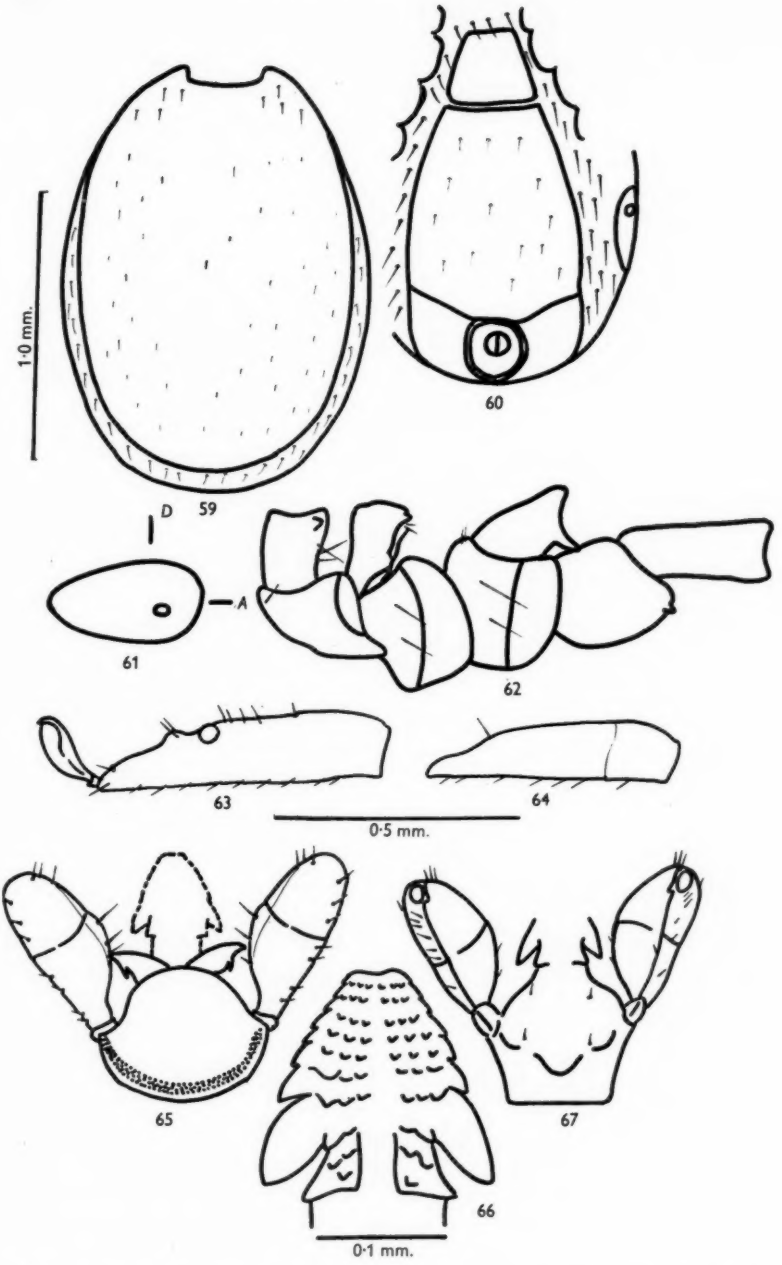
Colour brown, legs moderately long, long fine curved hairs on opisthosoma, being longest on epimeral plate and shortest on scutum. Length 2·1 mm., breadth 1·15 mm., broadly oval in shape.

Capitulum (Figs. 65, 66, 67). Basis capituli with broadly rounded dorsal ridge, slight angularity postero-laterally before continuing as the curved sides. Surface generally flattened with few faint punctations. Overall length 0·5 mm., breadth behind palpal insertion 0·34 mm. Palpi short, broad; slightly more than twice as long as broad, lateral profile almost straight, mesial profile convex, more so on article 2 than 3, article 2 broader than article 3, article 2 narrows rapidly to base, dorso-mesial edge flanged, length of article 2, 0·19 mm., article 3, 0·16 mm., breadth across apex of article 2, 0·15 mm. Ventrally sides of basis capituli slightly concave, diverge to palpal base from straight posterior edge, transverse ridge indicated by small 'auriculae' and a larger median lobe, weakly developed; surface in front of ridge gently convex, behind ridge almost flat, posterior face of ridge short, not declivitous, two pairs of hairs in position indicated in Fig. 67. Hypostome (Fig. 66), apex flat with mild indentation, lateral profile divergent to base; seven transverse rows of denticles in front of very large basal tooth, lateral teeth sharply pointed, mesial teeth as crenulations (4/4 files), basal tooth large, subtended by one transverse mesial row (2/2); sub-basal tooth marginally angled with irregular crenulations on its surface. Attachment of hypostome to basis broad. Length from basal tooth to apex, 0·19 mm.

Scutum (Fig. 59). Oval, length 1·6 mm., breadth at mid-length, 1·2 mm.; uniformly convex, covered with short fine hairs except anteriorly and antero-laterally where the hairs are longer, but not as long as those on marginal fold. Scapulae with rounded angles, most acute mesially; emargination moderate. Cervical grooves almost negligible, consisting of very short, shallow depressions. Punctations fine, uniformly distributed. Marginal fold relatively wide, fades out at level of second pair of coxae.

Ventral plates (Fig. 60). Pregenital plate with straight posterior edge, straight sides convergent anteriorly, front edge short and also straight; median plate 0·8 mm. long, maximum breadth near posterior extremity, 0·65 mm.; anal plate circular and drawn out to short point behind; adanal plates broader than long, extending only slightly in front of anal plate, mesial edge concave, lateral edges almost rectilinear but postero-lateral angles rounded; all with short fine hairs, punctations small, shallow but somewhat larger than on scutum; epimeral plates with longer and thicker hairs than elsewhere.

Legs. Of moderate thickness and length, coxae (Fig. 62) I–III with syncoxae, extensive in II and III, surface gently convex, supplied with long fine hairs, coxae IV overlapped by III, III by II and II by I, rather large, all with trenchant posterior edges; coxa I with large, broad, round-tipped internal spur, no external spur, spurs lacking on coxae II and III, short, broad, pointed spurs on coxae IV;

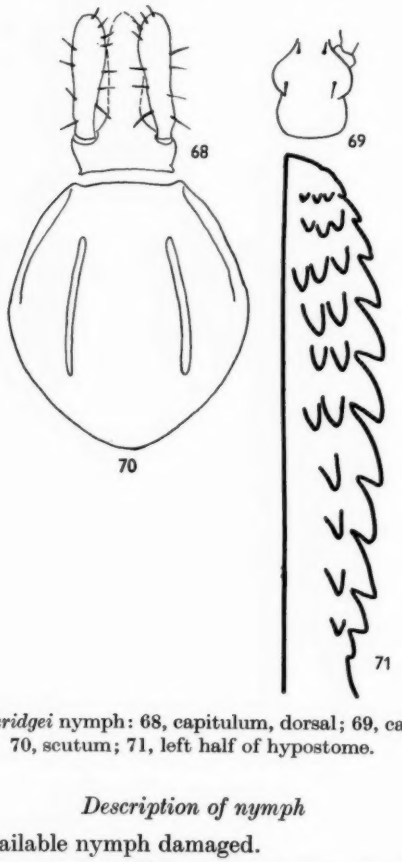


Figs. 59–67. *I. loveridgei*, male: 59, dorsum: 60, opisthosoma, venter; 61, spiracular plate; 62, coxae and trochanters I–IV; 63, tarsus I; 64, tarsus IV; 65, capitulum, dorsal; 66, hypostome; 67, capitulum, ventral. *A*, anterior; *D*, dorsal.

length of tarsus I, 0·58 mm., metatarsus I, 0·30 mm.; tarsus IV, 0·51 mm., metatarsus IV, 0·34 mm., all tarsi (Figs. 63, 64) taper distally with slight hump near apex before narrowing, pulvillus as long as claw, tarsi with hairs of moderate length. Trochanters I–III with spurs of increasing size, no trochanter spur visible on leg IV.

Spiracular plate. Long, piriform (Fig. 61), narrowing posteriorly, long axis parallel to that of body, macula anterior, goblets small, fairly numerous, dimensions 0·31 × 0·165 mm.

Genital opening. On a level with anterior third of coxae III.



Figs. 68–71. *I. loveridgei* nymph: 68, capitulum, dorsal; 69, capitulum, ventral; 70, scutum; 71, left half of hypostome.

Description of nymph

Dorsum of single available nymph damaged.

Capitulum (Figs. 68, 69). Overall length 0·32 mm., breadth 0·2 mm., dorsal ridge straight, drawn out postero-laterally to short sharp points. Surface convex, lateral margins slightly divergent to palpal insertion. Palpi club-shaped, no evident suture between articles 2 and 3; combined length of articles 2 and 3, 0·27 mm. Hairs long and numerous. Ventrally, posterior margin of basis capituli straight, constricted about midway along behind broadly rounded salient auriculae, sides of basis curved posterior to latter.

Hypostome. Curved profile, rounded apex; dentition from apex to base behind

a small corona as 3 rows 4/4 files, 3 rows 3/3 files, 5 rows 2/2 files. Lateral teeth more strongly pointed than median elements.

Scutum (Fig. 70). Broad, lateral carinae extending back to nearly half-way but not reaching margin; cervical grooves long, narrow, shallow depressions, indistinct anteriorly, not reaching postero-lateral border.

Legs. Coxa I with postero-internal salience as in female, external spur broad, coxae II–IV with external spurs only, short broad pointed trochanter spurs on legs I–III. Tarsi as in female.

Anal groove. Long, horseshoe-shaped. Body hairs abundant, long and fine.

Related species. This species has an affinity with the *rasus* and *ugandanus* group of species in the possession of a closed anal groove in both male and female. It is separated from the *ugandanus* group on the presence of the auriculae and from the *rasus* group (*I. pseudorasus* n.sp., *I. rasus* Neumann, *I. muniensis* n.sp., *I. procaviae* n.sp., *I. okapiae* n.sp. and *I. thomasae* n.sp.) on the shape of the auriculae, the form of the palps and the difference in size and orientation of the porose areas (Arthur & Burrow, 1957). The affinities of the female of this species also extend to *I. pilosus* Koch and to *I. lewisi* n.sp. in (1) not having external spurs on any coxae, (2) in having short indistinct cornua, (3) auriculae which are not retrograde, (4) palps which are indented laterally and in which there is a marked proximal protuberance on article II. *I. loveridgei* is, however, separable from the *pilosus* group of species in having the closed anal grooves (cf. with the horseshoe-shaped forms in *I. pilosus* and *I. lewisi*), palps which though indented are angled rather than concave, the presence of a meso-dorsal projection on palpal article 1, a difference in the shape of the dorsal aspect, in particular, of the basis capituli; in *I. loveridgei* the sides of the basis are very short compared with its length, whilst in the *I. pilosus* group the sides are much longer and the length of the basis relatively shorter. The males of *I. loveridgei* are also readily separated from those of the *I. pilosus* group on the shape of the dorsum of the basis capituli, which is more angular in the latter, on the form of the anal groove which is horseshoe-shaped in *I. pilosus* and *I. lewisi*, and in coxal shape and spurring as well as in the outline of the pregenital plate.

Hosts. Known only from *Cricetomys gambianus viator* Thomas.

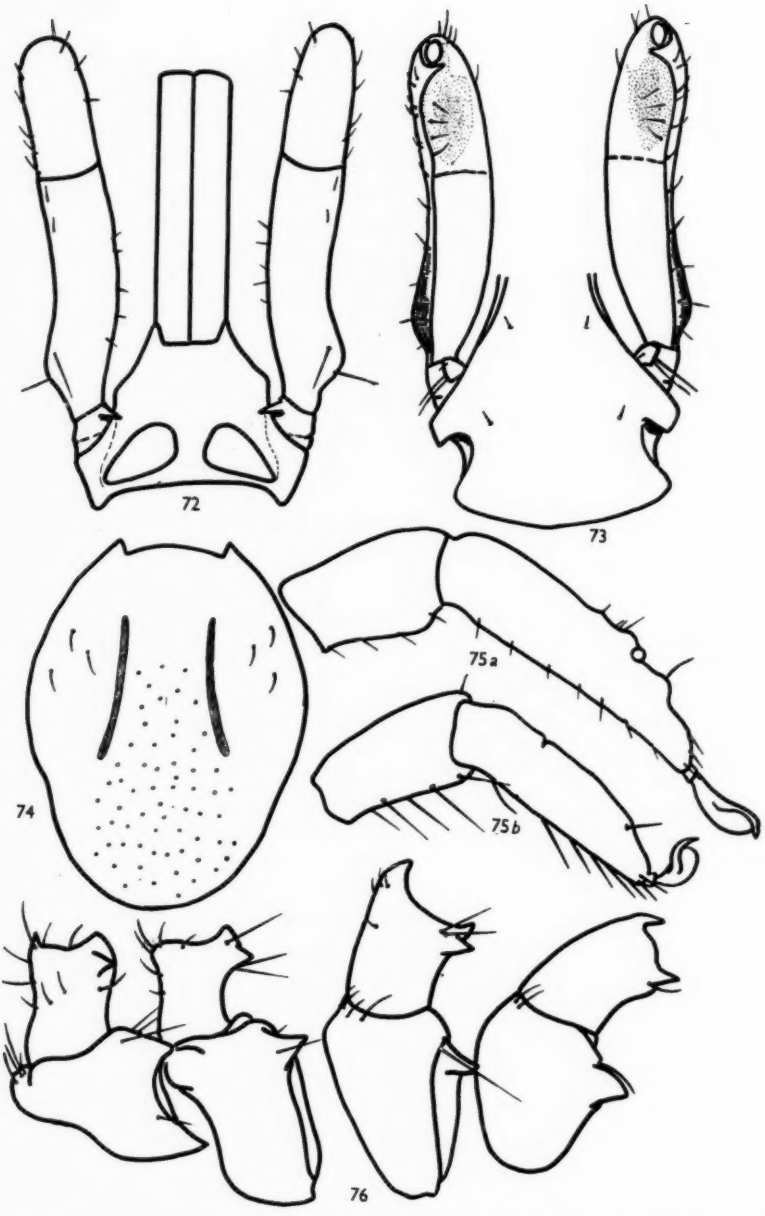
Distribution. Nyasaland.

Remarks. This species had been identified as *I. ampullaceus* Warburton by Bequaert, but there is little question that *I. ampullaceus* and *I. ugandanus* are synonymous. The species is named for Arthur Loveridge who has collected extensively in Africa.

Ixodes spinae n.sp. (Figs. 72–85)

Holotype. Female, *Procavia capensis*, Pietersburg, South Africa, 20. ix. 1954, deposited at the Onderstepoort Veterinary Research Department, Onderstepoort, South Africa.

Allotype. Male, *Procavia capensis*, Pietersburg, South Africa, 20. ix. 1954, deposited at the Onderstepoort Veterinary Research Department, Onderstepoort, South Africa.



Figs. 72–76. *I. spinae* female: 72, capitulum, dorsal; 73, capitulum, ventral; 74, scutum; 75*a*, tarsus and metatarsus I; 75*b*, tarsus and metatarsus IV; 76, coxae and trochanters I–IV.

Paratypes. 2 females with same data as above. Deposited at the Onderstepoort Veterinary Research Department, South Africa.

Description of female

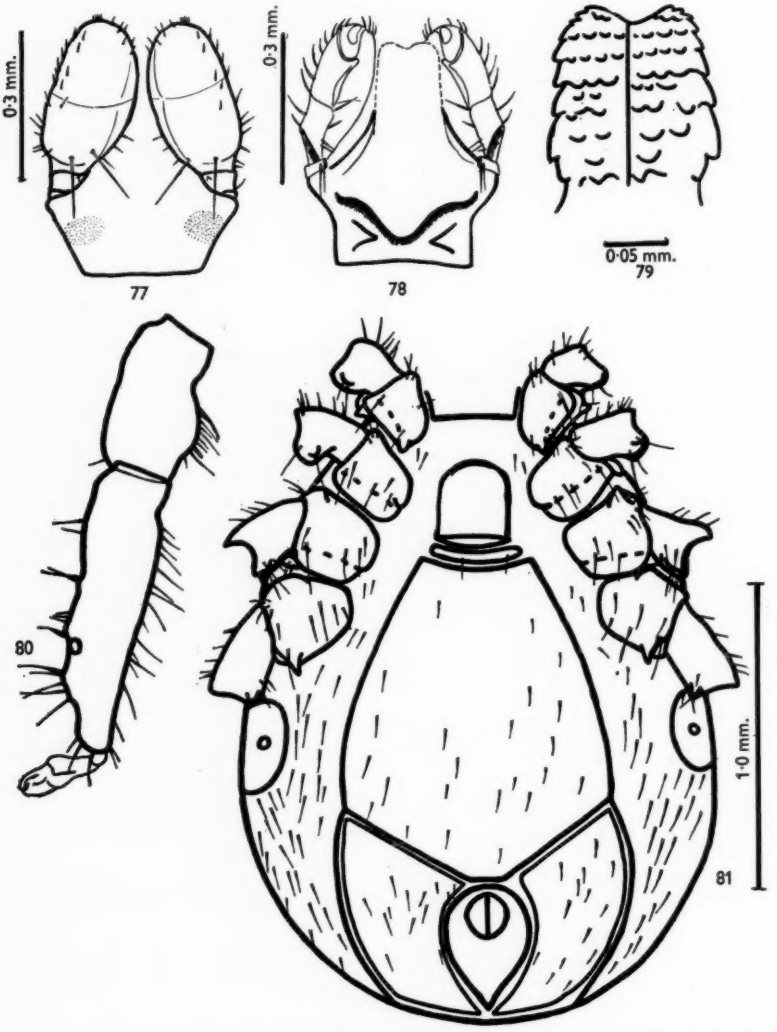
Large, colour in alcohol-preserved specimen dark brown, with long fine hairs on alloscutum; scutum and basis most heavily pigmented, legs and palps paler; scutum with few hairs. Engorged female oval in outline.

Capitulum (Figs. 72, 73). Length of basis capituli from dorsal ridge to margin of anterior cheliceral foramen 0·33 mm., breadth behind palpal insertion 0·47 mm.; posterior margin straight or slightly concave; cornua triangular, basal breadth greater than length, tapering to rounded apex; lateral margins relatively short (Fig. 72), diverging to palpal insertion thence converging strongly mesial to palpal base. Surface flattened with very slight downward curvature laterad (i.e. beyond the broken line in Fig. 72), of porose areas; latter slightly depressed, subtriangular in outline. Palpi long, narrow, about six times as long as broad; article 1 broader than long, with prominent pointed spur meso-dorsally (Fig. 72), and a shorter meso-ventral spur (Fig. 73); long hairs arise on each side of dorsal spur, a pair of longer hairs arising behind ventral spur; outer basal edge of article 2 moderately constricted basally, leading to slight swelling, thence to a concave outer margin; mesial margin slightly convex, width more or less uniform; article 3 with straight borders, apex rounded; hairs long and fine along outer profile of article 2, short mesially as on article 3. Ventrally, basis capituli divided into larger anterior and smaller posterior portion by shallow transverse groove, surface flat, base colour dark reddish-brown, posterior margin as a broad arc of a circle, postero-lateral angles sharply angled (almost pointed) (Fig. 73), lateral margins demi-concave extending forwards as far as inner base of auriculae, latter project postero-laterally, margins straight, angles sharp, ventral surface convex, slightly elevated above rest of basis capituli, posterior face declivitous (cross-hatched in Fig. 73), two pairs of hairs as in figure. Mesial face of palpal article 2 flat, distal portion of article 3 concave (stippled in Fig. 73). Hypostome: length 0·61 mm., apex rounded. Large number of teeth damaged in only specimen available, dentition would appear to be from apex to base as 7 rows 4/4, 4 rows 3/3, 7 rows 2/2, teeth long, narrow, pointed.

Scutum (Fig. 74). Length 1·38 mm., breadth 1·06 mm., elongate oval with sinuous postero-lateral margins, surface flat, hairs sparse, mostly very short, longest ones shorter than those on alloscutum; scapulae short, broad, tapering anteriorly, emargination moderate. Lateral carinae indicated by slight elevation, but generally indistinct. Cervical grooves weak anteriorly, apparent about mid-length as narrow, shallow depressions. Punctations small, shallow, predominate between cervical grooves and posteriorly.

Legs. Stout, moderate length, good cover of fine hairs of variable length; coxae I–III with narrow syncoxal areas, trenchant behind, surface convex with few coarse shallow punctations, long fine hairs, coxa I with broad, sharply pointed internal spur, external spur slightly shorter (Fig. 76), coxae II and III with strong pointed external spurs and short broad rounded internal spur located mesially of

postero-internal edge, coxa IV with broad, strong, pointed external spur, internal spur absent or appearing as a marginal salience; trochanter spur on legs I–IV strong, broad, moderate length, pointed; tarsi (Figs. 75*a*, *b*) broad, tarsus I slightly



Figs. 77–81. *I. spinae* male: 77, capitulum, dorsal; 78, capitulum, ventral; 79, hypostome; 80, tarsus and metatarsus I; 81, opisthosoma venter.

undulate dorsally, parallel-sided as far as Haller's organ, succeeded by a sharp hump, thence to a broad apical region, tarsus IV lacking such a hump; pulvillus almost as long as claw; hairs on tarsus and metatarsus IV spinose; length of tarsus I, 0·66 mm., metatarsus I, 0·36 mm., tarsus IV, 0·51 mm., metatarsus IV, 0·36 mm.

Genital opening. On a level with the interspace between coxae III and IV, covered by a large, almost rectangular genital apron; genital grooves divergent posteriorly becoming slightly rounded and convergent behind, not attaining posterior border.

Anal opening. Placed far back, grooves narrowly open behind in fed female; in unfed specimens elongate oval and drawn out to short point.

Spiracular plate. Oval, transverse to body axis, macula anterior of centre, goblets large.

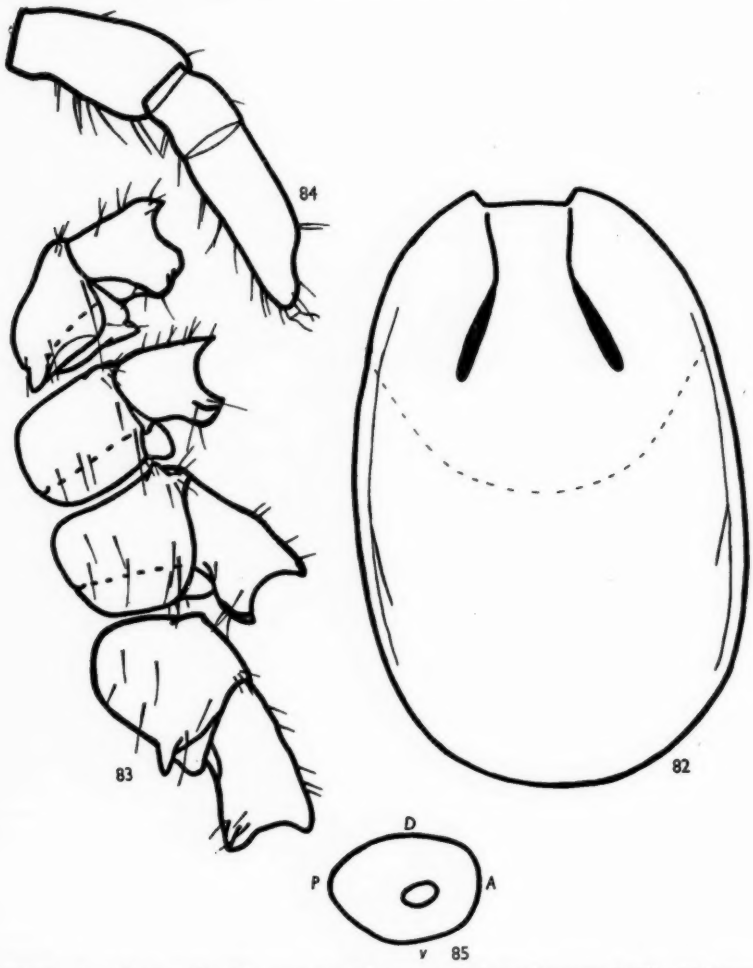
Description of male

Body large relative to female, oval but tapering more strongly anteriorly, broadly rounded posteriorly (Fig. 81). Greatest width on a level with spiracles. Colour in alcohol-preserved specimen: alloscutum brown with long adpressed white hairs, ventral plates reddish brown, legs, basis capituli and palpi reddish brown, scutum and coxae even more heavily pigmented.

Capitulum (Figs. 77–79). Overall length 0·27 mm., breadth across dorsal ridge 0·23 mm., base colour brown, most heavily pigmented peripherally except for a narrow paler band of colour along posterior edge. Posterior margin straight, lateral margins strongly divergent, straight to palpal base, greatest width where lateral margins attain palpal bases; cornua absent. Surface reticulately patterned, faintly convex between palps, shallow depressions about midway along basis capituli and nearer lateral border (indicated by stippling in Fig. 77). Palpi short, stout, about twice as long as broad, tapering gradually to a rounded apex, lateral and dorsal surface of article 2 swollen basally, with steep posterior curvature as it narrows strongly to the base (Fig. 77), mesial profile of articles 2 and 3 convex, length of article 2, 0·20 mm., article 3, 0·15 mm., greatest breadth at suture line between articles 2 and 3, article 1 broader than long, with very small pointed meso-dorsally directed spur, hairs numerous, moderate length, fine, particularly abundant laterally; a pair of longer hairs on each palp meso-dorsally near base of article 2. Ventrally, basis capituli about one and a third times as broad as long, posterior border of basis capituli straight, lateral margins straight for about half length of basis capituli, thence suddenly divergent to base of article 1; auriculae as small, heavily sclerotized edges directed ventro-laterally, transverse ridge between auriculae produced backwards in midline to form a broad tongue-shaped eminence, posterior face of ridge steep for short distance (cross-hatched in Fig. 78) then flat to posterior edge except for two triangular elevations, anterior surface of ridge strongly sloped to hypostome base. Palpal article 1 with strong-ridged ventrally directed projection and two hairs directed ventro-laterally; mesial surface of article 3 weakly indented, that of article 2 flat. Hypostome (Fig. 79) rises from basis capituli by broad, relatively long neck; length 0·15 mm., breadth across pre-basal teeth 0·15 mm., apex indented, lateral teeth large, pointed, mesial teeth as lobed crenulations, 9 lateral rows of teeth with irregular distribution of mesial crenulations as shown in Fig. 79.

Scutum (Fig. 82). Dark reddish brown colour, broadly ovate, length 1·88 mm., breadth 1·25 mm., with greatest breadth a little posterior of mid-length. Pseudo-scutal outline indistinct; cervical grooves short, almost parallel anteriorly, very

narrow, diverging to broader shallow depressions at about one-third the length of the pseudoscutum. Punctations numerous, equal, very fine, shallow, fairly uniform distribution over most of the scutal area, but less numerous between the scapulae, latter broad, blunt and subtriangular, emargination moderate. Hairs few, very short, white, predominating on the lateral margins. Marginal body fold of moderate width, narrowing at level of second intercoxal space.



Figs. 82–85. *I. spinae* male: 82, scutum; 83, coxae and trochanters I–IV; 84, tarsus and metatarsus IV; 85, spiracular plate. *A*, anterior; *D*, dorsal; *p*, posterior; *v*, ventral.

Legs. Stout, of moderate length; coxa I with small crescentic syncoxa, coxae II and III with larger syncoxae, coxa I trenchant behind with conical internal spur; coxae II and III with very broad flange-like posterior margins, coxa IV with long, narrow, pointed conical external spur, all coxae with moderate-sized deep puncta-

tions, hairs long and fine. Trochanters I–III with small flange-like saliences, trochanter IV with short conical pointed spur; tarsi with many medium-to-long hairs, length of tarsus I (Fig. 80), 0·57 mm., metatarsus I, 0·30 mm.; tarsus IV (Fig. 84), 0·50 mm., metatarsus IV, 0·35 mm.

Ventral plates. Adanal plates longer than broad, mesial margins concave ending in a point meso-posteriorly, lateral and posterior margins curved; median plate a little longer than broad, anterior edge short; pregenital plate parallel sided, broadly curved in front, all supplied with short white hairs; small shallow, uniformly distributed punctations on median and adanal plates. Hairs on epimeral plates long and fine.

Spiracular plate (Fig. 85). Large, oval with longitudinal axis parallel to that of body; dimensions 0·29 × 0·22 mm., macula antero-ventral, goblets large.

Anal groove (Fig. 85). Oval, tapering posteriorly, closed and drawn out to a point posteriorly; long axis parallel to that of body.

Genital opening. On level with anterior margin of coxae III.

Related species. I. spinae n.sp. is similar to most specimens of *I. ugandanus* Neumann 1906, and to some individuals of the *Ixodes rasus* complex of species in having an anal groove which is closed, circular and drawn out to a small posterior point (Arthur & Burrow, 1957). The long narrow palpi of this new species, which are more or less of uniform width along their length, also suggest an affinity with *I. ugandanus* and with at least some species of the *rasus* group. One salient difference between *I. spinae* and the aforementioned ticks is to be found in palpal article 1 which bears a prominent pointed spine dorsally in the former; this is without parallel in any other known African species of this genus. In addition each coxa of the females of *I. spinae* possesses a strong postero-external spur; this arrangement of spurs is lacking in the *I. rasus* group and *I. ugandanus*. Similarly, nothing comparable with the auriculae and ventral surface of the basis capituli of this new species is to be found in either of these groups.

Hosts. Known only from *Procavia capensis.*

Ixodes euplecti n.sp. (Figs. 86–91)

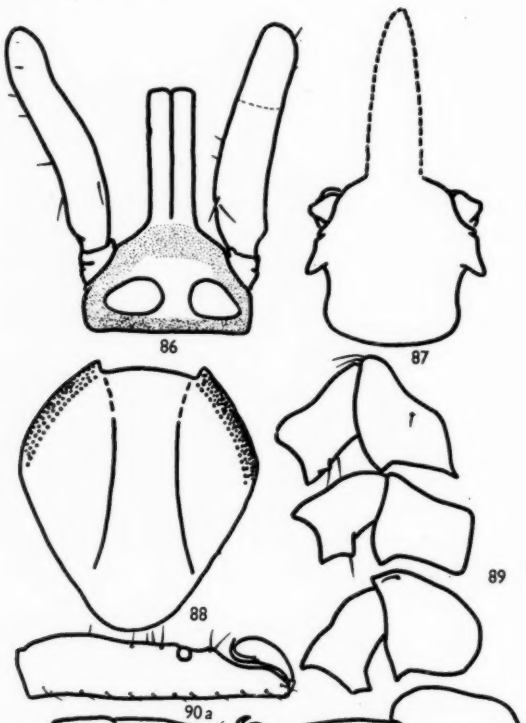
Holotype. Female, from male *Euplectus macrourus*, Bateke Pl., 2400 ft. alt., Moyen Congo, Djambala, French Equatorial Africa, 26. v. 1951. H. A. Beatty *leg.* Field no. H.A.B. 1101. Deposited in the Chicago Natural History Museum (W. Afr. Zool. Exped. 1950–2), cat. no. 213691. Known only from the holotype female.

Description of female

Small. Colour: alloscutum reddish brown, scattered short white hairs.

Capitulum (Figs. 86, 87). Surface flattened, central area pale yellow, margined by blackish brown pigmentation (stippled in Fig. 86). Length of capitulum 0·60 mm., breadth across dorsal ridge 0·32 mm., posterior margin straight, cornua lacking, lateral margins almost parallel. Porose areas transverse, egg-shaped; interporose interval narrow. Palpi of moderate length and breadth, about five times as long as broad, suture line between articles 2 and 3 indistinct, combined

length of articles 2 and 3, 0·45 mm., lateral profile of both articles concave, with corresponding convexity mesially, hairs longer on article 2 than 3. Ventrally basis capituli broad, very slight constriction just behind mid-length, postero-lateral angles rounded, posterior margin broadly curved; auriculae as sharp-edged pointed spurs (not retrograde). Surface flat, impunctate. Hypostome (Fig. 91), length 0·38 mm., narrowing from base to pointed apex; teeth long, slender, pointed, arranged from apex to base as 9 rows of 3/3, 5 rows of 2/2, 1 row of 1/1, with median triangular unarmed area.



Figs. 86–90*b*. *I. euplecti* female: 86, capitulum, dorsal; 87, capitulum, ventral; 88, scutum; 89, coxae and trochanters I–IV; 90*a*, tarsus I; 90*b*, tarsus IV.

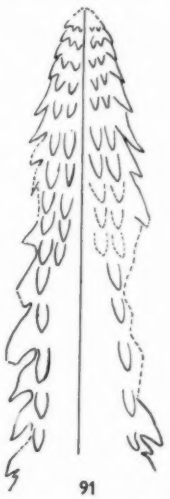
Scutum. Nearly as long as broad, length 1·0 mm., breadth 0·9 mm., widest in the anterior third, broadly curved antero-laterally, tapering rather sharply posteriorly, by rectilinear sides, narrowly rounded behind. Lateral carinae absent, antero-lateral margins downcurve quite strongly; cervical grooves narrow, shallow, depressions almost straight and extending nearly to posterior margins. Punctations as small, shallow depressions posteriorly but otherwise impunctate. Few short white hairs.

Legs. Of moderate length; coxa I triangular, postero-external spur short, broad, internal spur narrow, sharply pointed; coxa II rectangular with broad spur externally; external spur on coxa III short, sharp, similar spur on coxa IV; both these coxae have rounded mesial edges, all with very few, short hairs. Trochanter spurs very broad, broader basally than long, pyramidal in shape; tarsi long, tapering from proximal to distal end with slight hump beyond Haller's organ on tarsus I (Fig. 90*a*), tarsus IV with ill-defined hump (Fig. 90*b*); length of tarsus I, 0·54 mm., metatarsus I, 0·28 mm.; tarsus IV, 0·46 mm., metatarsus IV, 0·32 mm. hairs short.

Spiracular plate. Circular, macula anterior.

Anal groove. Elliptical, open, circular in front, lateral arms convex and converging posteriorly.

Related species. I. euplecti, since it lacks retrograde auriculae, has an internal spur only on coxae I and external spurs on all coxae, can be classified along with *I. rotundatus* new species, *I. daveyi* Nuttall 1913, *I. theilerae* Arthur 1953, *I. aubiensis* new species (Arthur & Burrow, 1957) and *I. spinae* new species. From *I. spinae* it is readily separated by the absence of a dorsal spine in article 1; from *I. rotundatus* by the absence of cornua, the ventral ridge on article 1 and the shape of the venter of the basis capituli (Fig. 2); from *aubiensis* in possessing trochanter spurs; from *I. theilerae* on basis, auriculae and palpal shape, and on hypostomal dentition amongst others, and from *I. daveyi* on hypostomal dentition (see Arthur, 1953), the absence of cornua and the longer, narrower external spurs on coxae I–IV of the latter.



91

Fig. 91. *I. euplecti* female, hypostome.

SUMMARY

The following new species of *Ixodes* from eastern Africa are described and figured: *I. rotundatus*, *I. auriculaelongae*, *I. nchisiensis*, *I. latus*, *I. loveridgei*, *I. spinae*, and *I. euplecti*.

The female of *I. oldi* is redescribed and the hitherto unknown male and nymph are described and figured. Some consideration is given to two specimens from Uganda which differ in certain particulars from typical specimens from East and West Africa.

REFERENCES

Arthur, D. R. (1953). *Ixodes theileri* n.sp., with observations on species confused therewith. *Parasitology*, **43**, 239–45.

Arthur, D. R. (1957*a*). The *Ixodes schillingsi* group: Ticks parasitic on Primates of Africa and Madagascar, with descriptions of two new species. *Parasitology*, **47**, 544–62.

Arthur, D. R. (1957*b*). Redescription of *Ixodes lunatus* Neumann, 1907 and of a new species confused therewith from Madagascar. *J. Parasit.* **43**, 474–83.

Arthur, D. R., & Burrow, C. D. (1957). The *Ixodes rasus* group of African ticks with descriptions of four new species. *Bull. Mus. comp. Zool.* (*Harv.*) **116**, 493–537.

Colas-Belcour, J. & Grenier, P. (1942). Sur un Ixodiné peu connu *Ixodes lunatus* Neumann 1907, ectoparasite des rats Malgaches. *Bull. Soc. Path. exot.* **35**, 54–65.

Nuttall, G. H. F. (1913). Notes on ticks. III. On four new species of *Ixodes*. *Parasitology*, **6**, 131.

(*MS. received for publication* 6. xi. 1956.—Ed.)

LIFE HISTORY AND PATHOGENICITY OF *EIMERIA ADENOEIDES* MOORE & BROWN, 1951, IN THE TURKEY POULT

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(With 6 Figures in the Text)

I. INTRODUCTION

The following seven species of *Eimeria* have been identified in turkeys in the U.S.A.: *E. meleagridis* Tyzzer, 1927, *E. meleagrimitis* Tyzzer, 1929, *E. dispersa* Tyzzer, 1929, *E. gallopavonis* Hawkins, 1950, *E. adenoeides* Moore & Brown, 1951, *E. innocua* Moore & Brown 1952, and *E. subrotunda* Moore, Brown & Carter, 1954.

E. adenoeides, the species discussed in this paper, was described as a new species by Moore & Brown in 1951. They obtained a pure strain of this coccidium by single oocyst isolation and described the morphology of the oocysts, experimental pathogenicity and localization in the intestine. The gross pathology of the experimental disease was studied, and the sporulation time and patent and prepatent periods established. These authors also confirmed the specificity of the parasite by cross-immunity tests with *E. meleagridis*. The life cycle of the coccidium could not be studied in detail as histological examinations were made from material collected only from the 5th to 7th day following the infection of poults, but their work did establish the fact that a distinguishing feature of *E. adenoeides* was that it invaded the deep glands as well as the tips of the villi. Since the prepatent period of *E. adenoeides* was found to be less than 5 days, Moore & Brown's work gave little information regarding the asexual stages of the life cycle, though they did describe 'intermediate forms' as being found in the same region as the sexual forms.

Moore & Brown (1951) reported that the mortality from infection with *E. adenoeides* may be 100% in birds up to 5 weeks of age, but that the death rate in poults over 7 weeks of age is very low, despite the prevention of previous accidental infection and the increase of the dose of sporulated oocysts to '10–20 times the lethal dose for poults under 5 weeks of age'. They did not state the number of sporulated oocysts used in their experiments but from their work there appears to be a definite age-resistance. This is in contrast to acute caecal coccidiosis in the chick caused by *E. tenella*, where resistance is dependent on infection and not on age alone (Horton-Smith, 1947). Moore & Brown also indicated that *E. adenoeides* caused reduced weight gains in older birds.

In Great Britain experimental coccidiosis in poults caused by 'suspected *E. adenoeides*' is mentioned briefly by Horton-Smith & Long (1956) in their paper on the control of histomoniasis by furazolidone.

The isolation and identification of a pure strain of *E. adenoeides* in the turkey poult in Great Britain was reported in a previous communication (Clarkson, 1956),

while in the present paper a detailed account is given of its life cycle and experimental pathogenicity.

II. MATERIALS AND METHODS

Birds. Three breeds of turkey poults were used—Beltsville White, Middle White and Broad Breasted Bronze. The majority were hatched in our own incubators but two lots were obtained as day-old poults. Daily faecal examinations were carried out from the 4th day by a sugar flotation technique to ensure that the poults were coccidia-free until they were experimentally infected with sporulated oocysts, usually at about 3 weeks of age.

Housing, feeding and management. Strict precautions were taken to prevent adventitious infection with coccidia. The birds were housed in a small locked room, the temperature of which was thermostatically controlled. Before the commencement of each experiment the room was washed out, the temperature set at 85° F. (29·4° C.), and the room fumigated with gaseous ammonia to destroy oocysts as described by Horton-Smith, Taylor & Turtle (1940).

The birds were reared in sheet metal cages, 2 ft. × 18 in. × 18 in., with a wire mesh floor through which the faeces fell to be collected on tarred paper for examination. The food and water troughs were arranged on the outside of the cage and were accessible to the birds by a slit in the metal sides. The cages could be dismantled to be cleaned and, together with all other equipment, were steam sterilized before use.

The attendants took great care to prevent extraneous infection by thoroughly washing the hands, discarding their boots and outer clothing before entering the room, and donning coccidia-free clothing and boots inside the room. The water used for the poults throughout the experiments was collected as hot tap water and kept in a covered sterilized bucket. From hatching to the age of 8 weeks the birds were fed on a proprietary brand of turkey starter pellets and from then onwards growers' pellets, both containing no drug supplements. The food was delivered directly into the room in unopened sacks from the suppliers.

Control birds were used in each experiment and were kept in the same room and under the same conditions as the infected poults. The faeces of the control birds were regularly examined and no accidental infection was ever detected, which suggests that the precautions taken were adequate.

Oocysts used to infect poults. Oocysts were obtained from clinical cases of coccidiosis in 3-week-old poults in which there was inflammation of the terminal small intestine, caeca and rectum, and histological sections showed infection of the deep glands of these regions, with oocysts of the same morphology as *E. adenoeides*. The intestinal contents of these poults were washed into large Petri dishes, mixed with 2·5 % potassium dichromate and the oocysts sporulated at 26° C.

A pure strain was obtained by selecting a single oocyst by means of a micromanipulator, and using it to infect a coccidia-free poult. The discharged oocysts were sporulated and passaged through further poults to obtain a large stock of oocysts of the same species. This technique has been described previously (Clarkson, 1956). All doses of oocysts except the single oocyst, which was placed in a

gelatine capsule, were fed directly into the crop by means of a sterile graduated syringe attached to a length of polythene tubing. The numbers of oocysts given were estimated approximately by the McMaster technique, and they were measured by means of a filar micrometer eyepiece under the $\frac{1}{12}$ in. oil-immersion objective.

Histology. In a pilot experiment on six poults sections were taken from the following eleven sites: across the two loops of duodenum, from the upper, middle and lower portions of the jejunum, ileum and caecum, and from the rectum. At no time were any stages of the parasite found in sections from the duodenum, jejunum, small intestine or upper ileum, and so in subsequent experiments sections were taken only from the other six sites.

All tissues were fixed within a few minutes after death in formol-saline saturated with mercuric chloride and were processed by the Nonex technique (Miles & Linder, 1953). Sections were cut at 3μ and stained either with Ehrlich's haematoxylin and eosin or Heidenhain's iron haematoxylin. Other staining techniques were also occasionally used.

Wet smears from all the regions examined histologically were fixed with methyl alcohol and stained with Giemsa by both the long and short methods, while other smears were fixed in Schaudinn's fluid and stained by Heidenhain's iron haematoxylin. Drawings of the stages of development of the parasite were made by means of a camera lucida, using the $\frac{1}{12}$ in. oil-immersion objective and a $\times 10$ eyepiece, giving a total magnification of 2000.

Haematology. Two experiments were carried out to investigate the effect of *E. adenoeides* infection on the blood picture of the poult. Red and white blood cell and thrombocyte counts were carried out using the technique described by Sadek (1955). Differential counts of the white blood cells were made in films stained by triple Leishman.

III. RESULTS

In a small pilot experiment six 3-week-old birds were each infected with 100,000 sporulated oocysts and were killed at 24 hr. intervals. From the findings in these birds it was decided in the main experiment to use a graduated dose depending on the phase of the life cycle when the bird would be killed, the largest doses being used for birds killed to show the early developmental stages.

In the main experiment two birds were killed at 1, 2, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 84, 90, 96, 108, 120, 132, 144, 168, 192, 216 and 240 hr. after infection to study the development of the parasite and the pathology of the disease. Three million oocysts were given to the birds killed from 1 to 54 hr. after dosing, 0·5 million to birds killed at 60–72 hr., 100,000 at 84–90 hr., 50,000 at 96–108 hr. and 10,000 to birds killed after this time. By adopting this graded dose many more trophozoites and 1st-stage schizonts could be seen and examined in each section. As the life cycle proceeds, asexual multiplication of the parasite gives rise to more numerous trophozoites in the succeeding stages. The birds were moved to sterile cages every day in an attempt to reduce the risk of re-infection by those sporulated oocysts from the original dose which pass unchanged through the intestine of the poult.

(*a*) *The life cycle*

Localization of stages in the intestine. The whole life cycle is passed in the terminal part of the small intestine, the caeca and the rectum, and the 1st asexual cycle is localized to a much smaller area than the 2nd asexual cycle, there being a successively greater spread as the parasite undergoes multiplication (Fig. 1). Thus the 1st asexual cycle occurs in the 'neck' of the caeca and in the terminal inch or so of the small intestine, whereas very few schizonts are found in the rectum. Second-stage schizonts occur throughout the caeca, and some are found in the small intestine and in the rectum. The sexual stage involves the whole of the caeca, the rectum and the posterior third of the small intestine. A few are found even more anteriorly, but none further forward than halfway to the yolk sac.

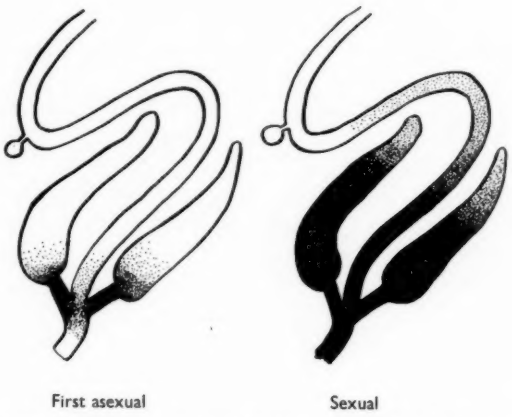

Fig. 1. Localization of first asexual generation and sexual generation of *E. adenoeides* in the intestine of the poult.

The sporozoites invade mainly the tips of the villi, but with some spread down the sides of the villi. They are only very rarely found in the deep glands, whereas the 2nd asexual generation occurs frequently in the deep glands and the sexual stages can always be found in the deep glands in large numbers as well as in all parts of the epithelium of the villi.

Sporozoites (Fig. 2A) were found in a bird given a massive dose of oocysts (3×10^6) and killed 2 hr. later. In this bird, sporulated and unsporulated oocysts could be found throughout the intestine, but were in largest numbers in the terminal small intestine and in the rectum, and the faeces showed large numbers. Sporocysts were also found in all sites, as were sporozoites, but the latter were relatively uncommon in the duodenum and upper small intestine, whereas the lower small intestine and rectum contained large numbers.

When examined on a warm stage the sporozoites exhibited several types of movement, including (*a*) contraction and elongation whilst remaining in one position, (*b*) sudden rapid gliding movements for a distance of 5 or 6 times their

own length, (*c*) curling and uncurling, and finally (*d*) a very peculiar movement with the blunt end apparently fixed and the pointed end circling around it.

The nucleus could be seen distending the sporozoite at its centre and this position of the nucleus was confirmed in smears stained with Giemsa. The cytoplasm was very pale blue, and the nucleus red, with red granules at the pointed end.

Sections of the lower small intestine showed these sporozoites in the lumen and in the act of entering the epithelial cells, whilst others had penetrated the cells and an occasional one could be seen below the cell nucleus, which is the normal site of development of the 1st-stage schizont. Sporozoites were seen in goblet cells as well as entering ordinary epithelial cells.

Six hours after infection, many of these early *trophozoites* were still elongated (Fig. 2B), while others had become rounded and still contained a single nucleus (Fig. 2C). The trophozoite grows rapidly and commences to undergo nuclear division to form the *1st-stage schizont*. In material taken as early as 12 hr. after infection occasional schizonts showed as many as 20 nuclei in the cytoplasm, but most schizonts showed only 4 or 5 (Fig. 2D). The schizont continues to grow, distends the parasitized epithelial cell, and undergoes nuclear division, so that in birds killed 36 hr. after infection most schizonts have 30–50 nuclei (Fig. 2E), although a few are less advanced in development. There is great variation in the degree of development of these schizonts, as shown by the size and number of nuclei present, and this wide range in the rate of development becomes more marked as the time interval from infection increases.

The nuclei of the schizonts consist of a deeply staining karyosome, eccentrically placed in a lighter staining material, presumably plastin, and surrounded by an unstained 'halo' of nucleoplasm (Tyzzer, 1929; Lapage, 1940).

The cytoplasm of the schizont, which may measure up to $25 \times 15\,\mu$ at 54 hr., becomes aggregated around the nuclei and up to 100 developing merozoites can be seen in a single sectioned schizont (Fig. 2F). An eosinophilic globule is present in the centre of all developing schizonts.

The mature 1st-stage schizont measuring $30 \times 18\mu$ and containing fully formed merozoites was first observed 60 hr. after infection (Fig. 2G). Serial sections of these schizonts showed that they contained some 700 merozoites. Six hours later almost all these merozoites had been released (Fig. 2H), but occasional large 1st-stage schizonts were found up to 84 hr. after infection. Thus the 1st asexual cycle is usually completed in $2\frac{1}{2}$–3 days.

The schizonts of many species of *Eimeria* have been reported as having a constant orientation in relation to the nucleus of the invaded epithelial cell, and in 80 out of 100 counted in this species the schizont was situated below the cell nucleus.

The *1st-stage merozoites* examined on the warm stage move in a different manner from sporozoites in that they exhibit a 'caterpillar-like' motion: they progress by contracting the central part upwards and then elongating again. In fresh preparations the nucleus can be seen as a bright object close to the centre of the merozoite and this is confirmed by its appearance in Giemsa-stained smears. These merozoites measure $4{\cdot}5$–$7{\cdot}0 \times 1{\cdot}5\mu$.

The 2nd asexual cycle is initiated by the invasion of epithelial cells by these

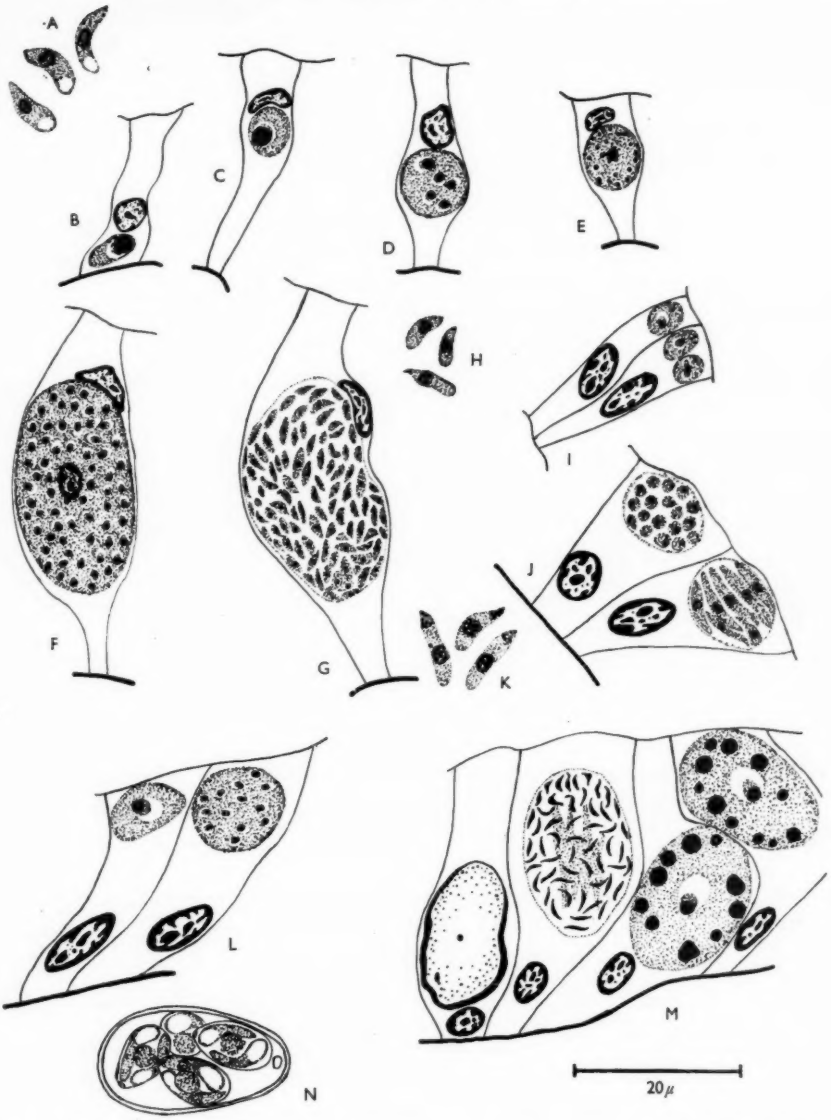

Fig. 2. Life cycle of *E. adenoeides*. A, sporozoites; B, sporozoite in cell, before becoming rounded off: 6 hr.; C, trophozoite: 6 hr.; D, E, developing schizont undergoing nuclear division: 12 and 36 hr. respectively; F, 1st-stage schizont: 54 hr.; G, mature 1st-stage schizont, containing merozoites: 60 hr.; H, 1st-stage merozoites: 72 hr.; I, developing 2nd-stage schizonts: 84 hr.; J, mature 2nd-stage schizonts containing merozoites: 108 hr.; K, 2nd-stage merozoites; L, early macro- and microgametocytes: 120 hr.; M, macrogametes, mature microgametocyte showing irregular residual mass and microgametes, and oocyst: 144 hr.; N, sporulated oocyst showing sporocysts containing sporozoites and residual body. A, H and K, Giemsa-stained smears; N, fresh preparation; remainder: sections stained with Heidenhain's iron haematoxylin (camera lucida, × 2000). The thick line represents the base of the epithelial cells.

1st-stage merozoites which become rounded and commence to undergo nuclear division (Fig. 2I), the nucleus having the same appearance as in the 1st-stage schizont. These *2nd-stage schizonts* are invariably above the cell nucleus and are only just beneath the brush border of the cell except where multiple invasion of a cell results in the deeper parasites being forced inwards. As many as four parasites have been seen in a single epithelial cell. The schizont does not grow as large as the 1st-stage schizont and the nuclei undergo fewer divisions.

Mature 2nd-stage schizonts, which measure $10 \times 10\mu$ and contain 12–24 large merozoites, may be found by the 96th hr. The majority of these schizonts are mature by 108 hr. (Fig. 2J) and thus the 2nd asexual cycle is completed in 4–4½ days.

The *2nd-stage merozoites* (Fig. 2K) are larger than the 1st, measuring about $10 \times 3\mu$, though there is considerable variation in size and shape. In Giemsa-stained smears the nucleus is slightly nearer the blunt end than the pointed end and numerous red granules are visible in the blue cytoplasm.

In birds killed 114 hr. after infection the 2nd-stage merozoites have invaded the epithelial cells, division of the nucleus has taken place, and the parasites have increased in size (Fig. 2L). These stages already represent the sexual cycle, but it is impossible to differentiate them from the early asexual stages; by 120 hr., however, the parasites can be differentiated into macrogametocytes, which are large and contain a large single nucleus, and microgametocytes which show many smaller nuclei (Fig. 2M). In smears the structure of the gametocytes can be seen clearly: their cytoplasm is granular, staining pale blue with Giemsa and grey with Heidenhain's iron haematoxylin, and the structure of the nucleus is quite distinct, showing the same features as in the schizonts. The microgametocytes cannot be differentiated from developing schizonts until the nuclei have divided many times, but their association with the macrogametocytes and their subsequent development leaves no doubt as to their identity.

The *macrogametes* (Fig. 2M) measure about $20 \times 18\mu$ and contain many large granules which stain as densely black with Heidenhain's iron haematoxylin as the karyosome, which is surrounded by achromatic material. The cytoplasm is granular. The large granules become situated at the periphery of the macrogametocyte and spread out to form the oocyst wall. The karyosome becomes much smaller and may not be visible at all.

The *microgametocyte* is approximately the same size as the macrogametocyte and at its surface lie numerous *microgametes*. It also contains a large irregular residual mass which usually shows long processes (Fig. 2M).

In many *Eimeria* species a 3rd asexual generation occurs (Tyzzer, 1929; Tyzzer, Theiler & Jones, 1932). Schizonts were found in our birds killed 5 and 6 days after infection, but were indistinguishable from the small 2nd-stage schizonts, and so the question arises whether they are delayed 2nd-stage schizonts or 3rd-stage schizonts. In the case of *E. tenella* the 3rd-stage schizont is easily distinguishable from the 2nd-stage by its size and in *E. necatrix* by size and also by the fact that the 3rd-stage schizonts occur in the caeca, whereas the 2nd-stage schizonts are found in the small intestine. In order to provide information on this point in the

case of *E. adenoeides*, two poults were inoculated with large numbers of sporulated oocysts, and the life cycle allowed to proceed until 2nd-stage merozoites were produced. The poults were then killed, one at 107 hr. and the second at 108 hr. after infection. The intestinal contents, which were rich in merozoites, were suspended in warm normal saline and injected *per rectum* into two coccidia-free poults, each receiving four injections over a period of 1 hr. This technique has been described by Tyzzer (1929), Levine (1940), Brackett & Bliznick (1952) and recently by Davies (1956). The birds were killed 24 and 28 hr. later and the caeca and terminal small intestine fixed and processed histologically. Stages in the development of the coccidium were found in several sections and examination of 200 of these stages showed that they were all gametocytes. This suggests that there is unlikely to be a 3rd asexual generation in *E. adenoeides* unless the proportion of asexual to sexual stages is very small.

The *prepatent period* was determined in thirty birds by collecting faeces at 2-hourly intervals, and examining for oocysts by sugar flotation. In this group two birds passed oocysts as early as 114 hr., whereas sixteen did not pass any oocysts until 132 hr. after infection, the latest prepatent period recorded. The distribution is shown in the histogram (Fig. 3).

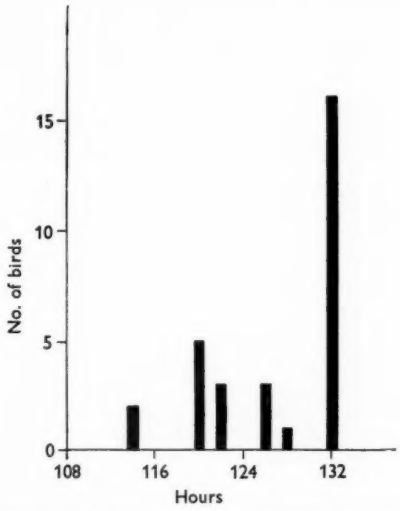

Fig. 3. Range of prepatent period in thirty poults (faeces collected at 2-hourly intervals).

The oocysts (Figs. 2N, 4) are ellipsoidal, the majority being symmetrical, but a few being more pointed at one end. When placed in 2·5 % potassium dichromate in an incubator at 26 ± 1° C. sporulation was complete within 24 hr. The fully sporulated oocyst shows one to three refractile granules at one end. The distribution of the length and width of 150 oocysts is shown in the accompanying histogram (Fig. 5), the average size being 25·6 × 16·25μ with an average L:W ratio of 1·58:1.

The *patent period* was determined on five poults. No oocysts were passed in any bird after the 20th day following infection and only very small numbers were passed later than 14 days after infection.

Fig. 4. Sporulated oocysts of *E. adenoeides*. × 470.

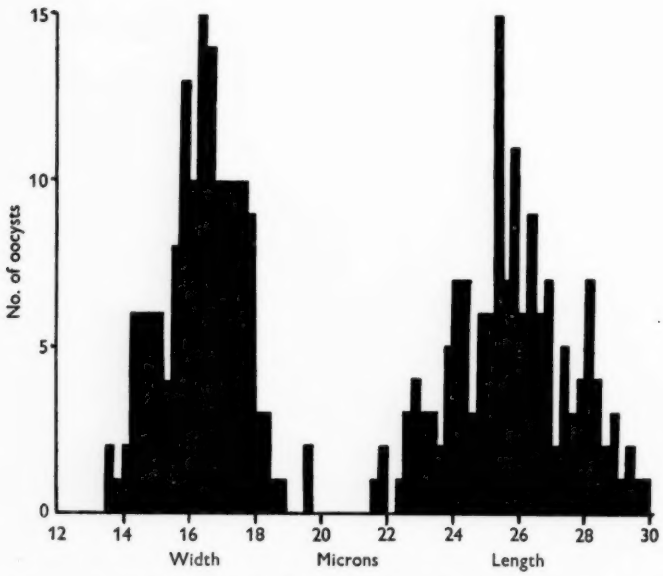

Fig. 5. Histogram of width and length of 150 oocysts of *E. adenoeides* in intervals of 0·25 μ.

(*b*) *Histology of the normal intestine*

Seven coccidia-free poults were used to study the normal histology of the different regions of the intestine and to provide material from birds of the same age range with which to compare experimentally infected intestines. In general the findings were similar to those reported by Demke (1954). Since the regions parasitized by *E. adenoeides* are the terminal small intestine, caeca and rectum. a brief account of their normal appearance is given.

The small intestine consists of an outer thin longitudinal muscle coat beneath the serosa and a much thicker circular coat followed by a very sparse submucosal layer. The muscularis mucosa is well developed and the intestinal glands are in direct contact with it. The crypts are quite short, but the villi are long and composed of connective tissue covered by simple columnar epithelium with a prominent brush border and many goblet cells.

The caecum differs in structure throughout its length. The narrow 'neck' or 'isthmus' is composed of the usual muscular layers, dense lymphoid tissue and villi with many goblet cells, and a very small lumen. The mid-portion has a large lumen and much thicker muscular walls, but they could not easily be differentiated into layers. The mucosa is arranged in large folds with smaller subsidiary folds, and contains many goblet cells. There is no brush border on the epithelial cells. Glands are present and there is a layer of fatty tissue between them and the muscular wall. The tip of the caecum is very similar to the mid-portion, but the folds are smaller. The rectum resembles the middle portion of the caecum, but the folds in the mucosa are very prominent and there are more subsidiary folds. These longitudinal folds were easily seen macroscopically when the rectum was opened. Goblet cells are very numerous.

No eosinophilic leucocytes were seen in sections of any part of the intestine, this being in agreement with Demke (1954) and an important observation in connexion with the pathology of *E. adenoeides* infection.

(*c*) *Symptoms and mortality*

Poults were quite normal for the first 3 days after infection and consumed the same amount of food and water as non-infected birds. On the 4th day they ate less food, the feathers became ruffled and the poults stood under the infra-red lamp with the head down, often between the legs, and cheeped plaintively. The faeces became very liquid and contained large amounts of mucus; towards the end of the 4th day and during the 5th day they were composed of orange-coloured mucus, small quantities of blood, and white mucus which contained enormous numbers of 2nd-stage merozoites, and a very watery material. As is mentioned by other workers on turkey coccidiosis, blood is not seen in large quantities, thus differing from *E. tenella* infection in chickens. During the 6th and 7th days the faeces were composed almost entirely of yellow cheesy 'casts' which were largely made up of immature gametocytes, oocysts and cellular debris, but subsequently the faeces became normal.

The symptoms described vary in severity according to the number of oocysts

ingested and dosage plays an important part in determining the mortality. Table 1 shows the mortality caused by administering different numbers of sporulated oocysts and, though the numbers of birds are small, the over-all effect can be seen.

Table 1. *Effect of age of poult and size of infective dose on mortality*

Age of poult (weeks)	Dose (no. of sporulated oocysts)	Mortality*
3	10,000	0/10
3	25,000	0/10
3	100,000	4/8
3	100,000	5/12
3	200,000	9/9
6	1×10^6	2/6
11	3×10^6	0/3

* Numerator: number of birds dying; denominator: number of birds dosed.

In the birds receiving 10,000 sporulated oocysts few symptoms were shown though the faeces were very liquid. In those fed 25,000 oocysts the symptoms were more marked and the birds ate nothing for 2 days, and, as will be seen later, considerable reduction in weight gain occurred as compared with the uninfected controls. When birds of 3 weeks of age were fed 100,000 sporulated oocysts the typical symptoms were observed in all of them and nine out of twenty died, on the 6th or 7th days. When birds of this age received 200,000 oocysts the mortality was 100 % and the deaths commenced late on the 5th day and before oocysts appeared in the faeces. Three coccidia-free birds 11 weeks old were each given an enormous dose of approximately 3 million oocysts, and although all showed the typical symptoms, including loss of weight, none died. This tends to support the conclusion of Moore & Brown (1951) that poults acquire an age-resistance to *E. adenoeides* which is not dependent on previous exposure to the coccidium.

Not only does *E. adenoeides* cause mortality in heavy infections but it also reduces the live-weight gains when smaller numbers of oocysts are given. Thus twenty-four poults of the same breed and hatch were kept under identical conditions and were weighed daily from hatching and divided into three groups of eight birds at 3 weeks of age by a random selection method which ensured that each group contained light, medium and heavy birds. Group I were uninfected controls, group 2 received 25,000 oocysts each and group 3 100,000 oocysts each. Food was given *ad lib.* throughout the experiment, and all three groups were kept in the same room, thus ensuring that the environment was constant. Weight gains and food consumption were recorded and the results are shown in Fig. 6 and Tables 2 and 3. The faeces of the control birds showed no oocysts for 4 weeks and these birds were therefore regarded as coccidia-free controls until 22 days from the commencement of the experiment. In the group which received 100,000 sporulated oocysts the 4 lightest birds died of coccidiosis on the 7th day, and thus this group had to be excluded from the comparison from that time. Since the range in weights of the 8 birds in each group was wide the mean weights were compared

statistically at 7, 14 and 21 days from infection and the results are presented in Table 2. Fig. 6 shows that the weight gains of both infected groups began to fall on the 6th day after infection. Parallel with these reductions in weight gain there was a fall in mean food consumption. Throughout the course of the experiment the group which received 25,000 oocysts consumed much less food and their conversion ratio over the 3-week period actually compared favourably with the uninfected birds (Table 3). A further point of interest which arises from this experiment is that the range in body weight of the eight birds in group 2 at the end of the

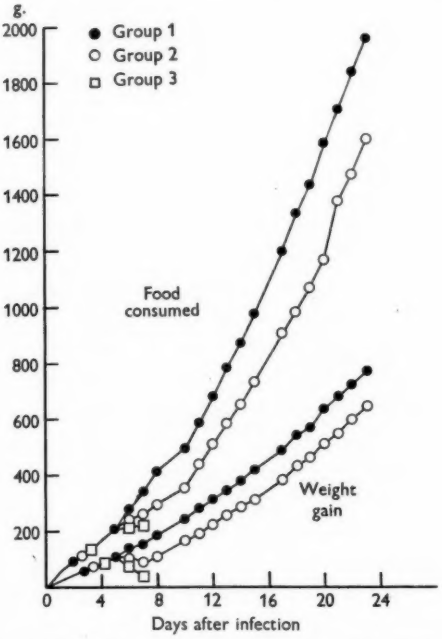

Fig. 6. Mean weight gain and food consumption of three groups, each of eight poults. Group 1: uninfected controls; group 2: infected with 25,000 sporulated oocysts; and group 3: with 100,000.

Table 2. *Effect of infection on weight gain (g.)*

Group (eight birds each)	Day of infection		7 days after infection		14 days after infection		21 days after infection	
	Mean wt.	Range	Mean wt.	Range	Mean wt.	Range	Mean wt.	Range
1. Control	226	200–251	381	306–414	606	455–702	907	679–1100
2. 25,000 oocysts	224	201–254	313 $P<0{\cdot}02$*	227–435	509 $P<0{\cdot}05$*	368–682	777 $P<0{\cdot}1$*	543–1026
3. 100,000 oocysts	226	200–250	268 $P<0{\cdot}001$*	219–321	†	†	†	†

* Significance of difference from control group at same age.

† Figures excluded due to death of four birds 7 days after infection.

experiment was greater than in group 1, the best birds being nearly as heavy as the best uninfected, which suggests that an attack of coccidiosis produces very uneven birds as well as mortality and reduction in weight gain.

Table 3. *Effect of infection on food consumption*

	Food conversion ratio [food (g.)/live weight (g.)]		
	0–7 days	0–14 days	0–21 days
1. Control	1·86	2·80	3·10
2. 25,000 oocysts	2·94	2·80	2·80
3. 100,000 oocysts	4·97	*	*

* Figures excluded due to death of four birds 7 days after infection.

(*d*) *Pathology*

Macroscopic. The intestine appeared quite normal until the 4th day after infection: on this day the birds went off their feed and so no food was found in the intestine, which became flattened; the lower part of the small intestine showed congestion, and the narrow part of the caeca became swollen and appeared oedematous. At the end of the 4th day the same picture was seen, but the intestine was now full of mucus which contained very large numbers of 2nd-stage merozoites; petechial haemorrhages, visible from the mucous surface but not from the serosal, were commonly found: they were particularly common in the narrow part of the caecum, the terminal part of the small intestine, and rectum.

During the 5th day in a heavy infection the greater part of the terminal intestine was congested and contained large numbers of merozoites and long streaks of blood; strands of orange-coloured mucus were found in the caecal orifices; at the end of the 5th day the intestine contained solid material of a yellow cheesy nature which was composed of cellular debris and gametocytes. Three birds died at this stage and no oocysts which were capable of sporulation were found in their intestines, though a small number of immature oocysts were seen in which golden-coloured contents filled the shell. In every other bird that died the intestine was congested and full of 'plugs' of oocysts, most of which were capable of sporulation.

The following is a typical post mortem on a 3-week-old bird dying on the 6th day of infection: the bird was in fair condition, but the skin was firmly adherent to the underlying tissues, no doubt due to the dehydration caused by the loss of water in the very liquid faeces; heart, liver, lungs and kidneys appeared normal; there was hardly any food material in the intestine, but the crop contained a small amount of whitish fluid. When the intestine was viewed from the serosal surface the duodenum and upper small intestine appeared normal, but the contents of the lower part of the intestine, including the rectum and caeca, were white, and the greatly enlarged blood vessels showed up clearly against them; petechial haemorrhages could also be seen through the serosa. On opening the intestine, the anterior half appeared normal except for lack of contents, but the posterior half was very abnormal. The terminal small intestine and rectum were filled with a yellow-white mucoid material which was composed largely of oocysts, with a small amount of cellular debris and red blood cells. The narrow part of the caeca also

contained this material, but in the wide part the contents were darker brown and contained 'clots' of a yellow cheesy material. The contents of the caeca varied quite considerably in different birds, even when they had received the same number of oocysts and died on the same day: some showed the normal contents mixed with a few cheesy clots, others contained a yellow 'cast' which was moulded to the folds of the caecum, and the remainder showed graduations between the two extremes.

On the 6th–8th days, in birds infected with 10,000 oocysts, the terminal intestine contained a white creamy mucus, and petechial haemorrhages were visible only from the mucosal surface. By the 9th day the intestinal contents appeared to be normal, though they still contained large numbers of oocysts, and small clots of solid material were still present in the caeca.

Microscopic. The most striking and rapid response of the host tissues to the invasion of *E. adenoeides* is infiltration of eosinophilic leucocytes. As mentioned previously, these were not present in the intestine of coccidia-free birds, but as early as 2 hr. after a massive infection large numbers of eosinophilic leucocytes were found in the submucosa and many had migrated between the epithelial cells. These leucocytes were not localized to the regions which were parasitized but were present in all sections taken from the duodenum to the rectum. They were found in these sites throughout the first $2\frac{1}{2}$ days of infection, but from the 3rd day they appeared to become more localized to the terminal small intestine, caeca and rectum. They were present in enormous numbers in these regions until 10 days from infection, the period studied in this experiment, and were also present in two birds which were infected with 2nd-stage merozoites *per rectum*.

Apart from this infiltration with eosinophilic leucocytes, no changes were seen until 4 days after infection and these were associated with the presence of stages of the coccidium and therefore were localized to the posterior part of the intestine. In birds killed 96 hr. after infection the muscle fibres of the wall of the narrow part of the caecum were separated, and spaces, presumably distended with fluid, were visible, thus confirming the oedematous appearance of this region visible macroscopically. This oedema was present until the 7th day after infection; the epithelial cells were reduced in size, being cubical rather than columnar, and—particularly at the tips of the villi—were packed with schizonts. In some places the epithelial tips of the villi had broken off, releasing 2nd-stage merozoites, cellular debris, eosinophilic leucocytes and red blood cells into the lumen.

After 108 hr. the picture was similar, but the blood vessels were engorged and there was an increased cellularity of the submucosa. After the release of the 2nd-stage merozoites the epithelial cells were rapidly replaced and the only pathological changes seen at 5 days were the oedematous muscle coats, increased vascularity and the presence of numerous round cells in the submucosa. Six days after infection the epithelial cells contained as many as five gametocytes each and in many sites the cells at the tips of the villi were removed, exposing the connective tissue core and large blood vessels, though these did not appear to be ruptured. In birds which died at this stage the increased vascularity involved vessels in the serosa, those between the muscle layers and long vessels extending

into the villi as far as their tips. The lumen of the intestine was packed with oocysts and with cellular debris from the tips of the villi, many of which were completely denuded of epithelium.

In birds which recovered from the disease or which had received only small doses of oocysts, the resolution of the intestine was very rapid, so that by 7 days from infection the deep glands were almost clear of gametocytes and the vascularity was greatly reduced. The submucosa contained large numbers of small round cells, particularly at the base of the villi, and the epithelium of the villi was composed of two or three layers of small cells instead of a single layer of columnar cells. Eight days from infection repair had proceeded further and the epithelial cells were normal, apart from the presence of small numbers of gametocytes, but the small round cells in the submucosa had become so numerous that many of the deep glands were surrounded and isolated from the lumen of the intestine. Some of these glands had become greatly dilated, while others were packed with necrotic tissue consisting of oocysts, glandular epithelium and red blood cells. On the 9th and 10th days after infection there were very few parasitic stages left and the tissues were almost normal except for isolated glands with small flattened cells containing necrotic tissue in their lumen.

(*e*) *Haematology*

As the host reacted so strikingly to infection with *E. adenoeides* by infiltration of the intestines with eosinophilic leucocytes, experiments were carried out to see if this was reflected in the constituents of the blood.

Blood samples were taken daily from two coccidia-free birds, two birds infected with 25,000 oocysts and two with 100,000. At no time during the course of infection were significant differences noted in the blood picture, which included total red and white blood cell counts, and thrombocyte and differential leucocyte counts.

As the eosinophilic response in the tissues was marked by 2 hr. after infection it was decided to investigate the haematology over the first 6 hr. of infection, by taking blood at hourly intervals in three control and three infected birds. Once again no significant differences attributable to the coccidial infection were found.

IV. DISCUSSION

The most important point for discussion is the evidence which identifies this coccidium as *E. adenoeides*. Tyzzer (1928) laid down the criteria which should be applied when describing new species of coccidia and these must also be used when identifying a species from accounts by other workers. All descriptions of the species of coccidia in turkeys have been made in the U.S.A., and from the sporulation time, localization in the intestine and morphology of oocysts the species described in this paper is either *E. meleagridis*, *E. gallopavonis* or *E. adenoeides*.

The prepatent periods of these three species are 5 days, 6 days and 112 hr. respectively (Moore, 1954); the prepatent period of our species in thirty birds ranged from 114 to 132 hr., which covers the range of all three species. Edgar

(1955) states that the prepatent period of *E. adenoeides* is 104 hr., but gives no details of the method used to determine this period or the range of the results. Andrews (1926) first used the term 'prepatent period' and defined it as 'the time from the entrance until the appearance of the parasites in the blood or faeces as determined by a stated method'. Tyzzer *et al.* (1932) point out that the appearance of merozoites in the faeces would fit this definition and therefore did not use the term, but it is now agreed that in the study of the coccidia it refers to the period from the entrance of sporulated oocysts into the host to the appearance of oocysts in the faeces. Moore (1954) suggests that it should refer to the appearance of *viable* oocysts as judged by collection, sporulation *and* infection of poults. Henry (1932) states that the normal prepatent period may vary from 12 to 24 hr. and suggests that factors which influence this period are age of the host and resistance due to previous infection. Andrews (1926) found that the prepatent period of *Isospora felis* in the kitten decreased as the age of the animal increased, but stated that the very short prepatent periods may have been due to the delayed passage of oocysts retained from the experimental infection.

In our experiments twenty-five of the poults were of the same breed and age and the oocysts used for infection were recently passaged and thus these factors could not explain the range observed, and five older birds showed almost the same range. The number of oocysts used to infect the bird, except for the single oocyst which gave a long prepatent period of 132 hr., did not influence the prepatent period, nor was it altered significantly by feeding oocysts to birds with empty crops. It would seem then that this range of prepatent period is normal, and that the prepatent period should be defined as the *minimum* time elapsing before oocysts are passed in a group, preferably with some indication of the range.

Andrews (1926) defined the patent period as 'the time elapsing from the appearance to the disappearance of parasites in the blood or faeces' though other workers have also included in it the prepatent period (Moore & Brown, 1951). In any case it appears to vary over several days and seems to be of little value in determining specificity (Moore, 1954).

The life cycle described in this paper is of the same general pattern as in other species of *Eimeria* in any host, but the morphology of the stages in the tissues differs from any previously described in the turkey.

Tyzzer (1929) states that the schizonts of *E. meleagridis* are large and possess a residual mass, the latter being a distinguishing feature. Hawkins (1952) describes both 1st- and 2nd-stage schizonts of this species as being small and containing twelve to fourteen merozoites, only the 2nd-stage schizont containing a residual mass. The 1st- and 2nd-stage schizonts develop above the nucleus of the epithelial cell, whereas many gametocytes develop deep below the cell nucleus. Hawkins (1952) described the stages of *E. gallopavonis*, but his description is incomplete in that no birds were killed earlier than 3 days from infection. The 1st-stage schizont was small, containing eight merozoites, possessed a residual mass and developed above the cell nucleus. There appeared to be two types of 2nd-stage schizont, one very small and superficially situated and a second which was large, deep below the cell nucleus and contained a 'large undetermined number of merozoites', this

schizont being found in the rectum only. The 3rd generation, both sexual and asexual, develop above the cell nucleus.

The morphology of the asexual stages of *E. adenoeides* has not been described and thus no comparison can be made with our species. We found a large 1st-stage schizont containing several hundred merozoites and lying deep to the cell nucleus, a 2nd-stage schizont which is small and placed superficially, and a 3rd generation, which is sexual, and is also above the cell nucleus. The large 1st-stage schizont is very similar to the 2nd-stage schizont of *E. gallopavonis* described by Hawkins, but as this was confined to the rectum it does not appear to be the same as ours. Moore & Brown (1951) describe *E. adenoeides* gametocytes as developing in the deep glands, whereas *E. meleagridis* and *E. gallopavonis* develop in the tips of the villi. Our species shows the deep site of development. *E. meleagridis* is not considered to be pathogenic (Moore & Brown, 1951; Hawkins, 1952; Demke, 1952) and Hawkins (1952) states that further study is required to determine the pathogenicity of *E. gallopavonis*. In contrast, *E. adenoeides* is very pathogenic to poults under 5 weeks old, but less pathogenic in older birds (Moore & Brown, 1951).

Our strain of this species is also very pathogenic and an age-resistance develops which is not dependent on previous infection. It is the sexual stage of the life cycle which is pathogenic, as most birds die on the 6th day while passing oocysts, although with large doses of oocysts death occurs before the prepatent period has elapsed and large numbers of 2nd-stage merozoites are still present in the intestinal contents but sections show enormous numbers of developing gametocytes. From this evidence (Table 4) our parasite can be identified as *E. adenoeides*. It was desired to confirm this by using cross-immunity tests on a strain which was known to be *E. adenoeides*, but there does not appear to be a viable strain in the U.S.A. (Moore, 1956).

E. adenoeides did not produce any effect on the blood picture of the infected poult. In the case of the red blood cell count this is not surprising, as severe haemorrhage was not associated with the experimental disease, unlike the effect of *E. tenella* in the chicken (Natt & Herrick, 1955). In infected birds the tissues rapidly became infiltrated with eosinophilic leucocytes and it would seem probable that this would be reflected in the blood picture. In sections some of these leucocytes contained small spherical granules while others possessed rod-like granules, and thus it was impossible to decide if this infiltration was due to heterophiles or true eosinophiles. However, in stained smears no changes attributable to *E. adenoeides* infection could be detected in either heterophile or eosinophile counts. This may have been due to inaccuracy in the methods employed or to the fact that the tissue response was not great enough to be reflected in the blood. Nonex embedded tissues were used for histological study throughout this work and produced good results in that the shrinkage around the parasites was negligible.

E. adenoeides has thus been confirmed by our experiments to be highly pathogenic for young poults, producing a mortality rate of 50–100 % in 3-week-old birds given 100,000–200,000 oocysts. A smaller dose had a significant effect on weight gain, but little or none on food conversion rate. During the course of the experimental work a number of outbreaks of coccidiosis in the field were brought to our notice.

Examination of poults from several of them confirmed the findings reported in the experimental study of the species, and there is no doubt that *E. adenoeides* causes losses in poults under natural conditions in this country.

Table 4. *Characteristics of* Eimeria adenoeides (*British and American*), E. gallopavonis *and* E. meleagridis

Characters		Species...	*E. adenoeides* British	*E. adenoeides* American	*E. gallopavonis*	*E. meleagridis*
Oocyst	Size (μ)	Length	21·50–30·00	18·95–31·26	22·2–32·7	20·3–30·8
		Width	13·50–19·50	12·60–20·94	15·2–19·4	15·4–20·6
		Mean	25·68 × 16·25	25·60 × 16·59	27·1 × 17·2	24·4 × 18·1
	Shape		Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal
	Refractile granule		Present	Present	Present	Present
	Sporulation time (hr.)		24	24	24	24
Localization in intestine	—		Lower ileum, caeca and rectum: deep glands as well as villi	Lower ileum, caeca and rectum: deep glands as well as villi	Rectum, ileum and caeca: tips of villi	Caeca, ileum and rectum: tips of villi
Morphology of stages and site in epithelial cell	Asexual	1st	Large, below cell nucleus	?	Small, above cell nucleus	Small, above cell nucleus
		2nd	Small, above cell nucleus	?	(*a*) Small, above cell nucleus (*b*) Large, below cell nucleus in rectum only	Small, above cell nucleus
	Sexual		Above cell nucleus	?	Above cell nucleus	Above and below cell nucleus
Prepatent period	—		114–132 hr.	104, 112 hr. approx.	144 hr. (6 days)	119 hr., 120 hr. (5 days)
Pathogenicity	—		+ + +	+ + +	?	±

V. SUMMARY

The life cycle and pathogenicity of a strain of *Eimeria* isolated in Great Britain from turkey poults by single cell inoculation are described and, using the criteria laid down by Tyzzer, the species is identified as *E. adenoeides*.

The life cycle is of the same general pattern as in other *Eimeria* species, consisting of two asexual and one sexual generations.

The organism is highly pathogenic for young poults, a dose of 200,000 oocysts producing 100 % mortality in 3-week-old birds and smaller doses causing reduced weight gain. Birds 11 weeks old resisted a dose of 3 million oocysts.

The gross and microscopic pathology of the infection is described. No changes were found in the blood picture.

I should like to thank Professor E. G. White for his guidance throughout the work and advice in preparation of the manuscript and Miss Moya Gentles for technical assistance. I am also grateful to Dr C. A. Hoare, F.R.S., for valuable advice at the manuscript stage. This work was carried out with the aid of a grant from the Agricultural Research Council.

REFERENCES

ANDREWS, J. M. (1926). Coccidiosis in mammals. *Amer. J. Hyg.* **6**, 784–98.

BRACKETT, S. & BLIZNICK, A. (1952). Relative susceptibility of chickens of different ages to coccidiosis caused by *E. necatrix*. *Poult. Sci.* **31**, 146–8.

CLARKSON, M. J. (1956). Experimental infection of turkey poults with *Eimeria adenoeides* isolated from a natural case in Great Britain. *Nature, Lond.*, **178**, 196–7.

DAVIES, S. F. M. (1956). Intestinal coccidiosis in chickens caused by *Eimeria necatrix*. *Vet. Rec.* **68**, 853–7.

DEMKE, D. D. (1952). Cecal coccidiosis in turkey poults. M.Sc. Thesis for the A. and M. College of Texas.

DEMKE, D. D. (1954). A brief histology of the intestine of the turkey poult. *Amer. J. vet. Res.* **15**, 447–9.

EDGAR, S. A. (1955). Sporulation of oocysts at specific temperatures and notes on the prepatent period of several species of avian coccidia. *J. Parasit.* **41**, 214–16.

HAWKINS, P. A. (1950). Coccidiosis in turkeys. Ph.D. Thesis, Michigan State College, East Lansing.

HAWKINS, P. A. (1952). Coccidiosis in turkeys. *Tech. Bull. Mich. St. Coll.* no. 226.

HENRY, DORA P. (1932). Coccidiosis of the guinea pig. *Univ. Calif. Publ. Zool.* **37**, 211–69.

HORTON-SMITH, C. (1947). Coccidiosis—some factors influencing its epidemiology. *Vet. Rec.* **59**, 645–6.

HORTON-SMITH, C. & LONG, P. L. (1956). Furazolidone in the control of blackhead in turkeys. *J. Comp. Path.* **66**, 22–4.

HORTON-SMITH, C., TAYLOR, E. L. & TURTLE, E. E. (1940). Ammonia fumigation for coccidial disinfection. *Vet. Rec.* **52**, 829–32.

LAPAGE, G. (1940). The study of coccidiosis in the guinea pig. *Vet. J.* **96**, 144–54, 190–202, 242–54, 280–95.

LEVINE, P. P. (1940). The initiation of avian coccidial infections with merozoites. *J. Parasit.* **26**, 337–43.

MILES, A. E. W. & LINDER, J. E. (1953). Polyethylene glycols as histological embedding media. *J. R. Micr. Soc.* **72**, 199.

MOORE, E. N. (1954). Species of coccidia affecting turkeys. *Proc. 91st Annual Meeting*, Amer. Vet. Med. Ass. pp. 300–3.

MOORE, E. N. (1956). (Personal communication.)

MOORE, E. N. & BROWN, J. A. (1951). A new coccidium pathogenic for turkeys, *Eimeria adenoeides* n.sp. *Cornell Vet.* **41**, 125–36.

MOORE, E. N. & BROWN, J. A. (1952). A new coccidium of turkeys, *Eimeria innocua* n.sp. *Cornell Vet.* **42**, 395–402.

MOORE, E. N., BROWN, J. A. & CARTER, R. D. (1954). A new coccidium of turkeys, *Eimeria subrotunda* n.sp. *Poult. Sci.* **33**, 925–9.

NATT, M. P. & HERRICK, C. A. (1955). The effect of caecal coccidiosis on the blood cells of the domestic fowl. I. *Poult. Sci.* **34**, 1100–6.

SADEK, S. E. (1955). A simple direct technique of counting avian blood cells. *J. Amer. Vet. Med. Ass.* **127**, 72–3.

TYZZER, E. E. (1927). Species and strains of coccidia in poultry. *J. Parasit.* **13**, 215.

TYZZER, E. E. (1928). Methods for isolating and differentiating species of *Eimeria* occurring in gallinaceous birds. *J. Parasit.* **15**, 148–9.

TYZZER, E. E. (1929). Coccidiosis in gallinaceous birds. *Amer. J. Hyg.* **10**, 269–384.

TYZZER, E. E., THEILER, H. & JONES, E. E. (1932). Coccidiosis in gallinaceous birds. II. A comparative study of species of *Eimeria* in the chicken. *Amer. J. Hyg.* **15**, 319–93.

(*MS. received for publication* 5. III. 1957.—Ed.)

NOTES ON THREE SPECIES OF *PTERYGOSOMA* (ACARINA: PTERYGOSOMIDAE) FROM GERRHOSAURID LIZARDS

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(With 3 Figures in the Text)

This is the first of a proposed series of papers on the Prostigmatid mites of lizards. The mites have been collected from lizards in the British Museum (Natural History), London, and I would like to acknowledge the assistance given by the members of the Museum Staff in the Reptile Section. I would also like to thank Dr G. Owen Evans and Mr E. Browning for their assistance and ready advice; The Director and Mr A. J. Hesse, of the South African Museum, Cape Town, for making available the type material of *Pterygosoma bicolor* Law, 1935, and *P. gerrhosauri* Law, 1935; Miss B. P. Wiltshire for typing the manuscript; and finally Dr F. A. Turk for reading the manuscript and for his advice and encouragement.

Lawrence (1935) has investigated the ectoparasites of lizards of the family Gerrhosauridae, and has found them to harbour three species of *Pterygosoma*. I have collected two of these species.

In the following discussion on these species the smaller anal setae, or spines, between the larger more obvious anal setae are referred to as the 'anal spines'.

Pterygosoma hystrix Lawrence, 1935

The five specimens of this species, from *Gerrhosaurus flavivulgaris* (locality unknown), show differences from the original description, which are important enough to be put on record since they add to our knowledge of the variation found in the species.

There is a fine, simple seta near to the insertion of trochanter II on the anterior common coxal plate. It is subequal in size to its two neighbouring coxal setae. The posterior common coxa possesses the usual two setae.

The dorsal setae on palp segments I and II are weakly serrate. The setation of the palp tarsus is shown in Fig. 1*a*.

DIMENSIONS:

Length (μ)	707	727	645	666
Breadth (μ)	1095	1053	1058	1109
$\frac{\text{Breadth}}{\text{Length}}$	1·55	1·45	1·64	1·66

The type measured 1100μ long by 1530μ broad. There is no record of the range in size.

Pterygosoma gerrhosauri Lawrence, 1935

This species has been collected off *Gerrhosaurus validus* and *G. major grandis*, but specimens of these lizards examined in the British Museum collections were not parasitized by Pterygosomidae.

The following observations on the females from the type material may prove useful in conjunction with the original description. There are three pairs of small, fine, simple setae on the ventral body surface and usually four pairs of anal spines between the anal setae. Palp segment I has an obvious, slender, simple seta near to the anterior ventral margin of the segment. This material also contained specimens of males, protonymphs, deutonymphs, and a larva, which I take the opportunity of describing for the first time.

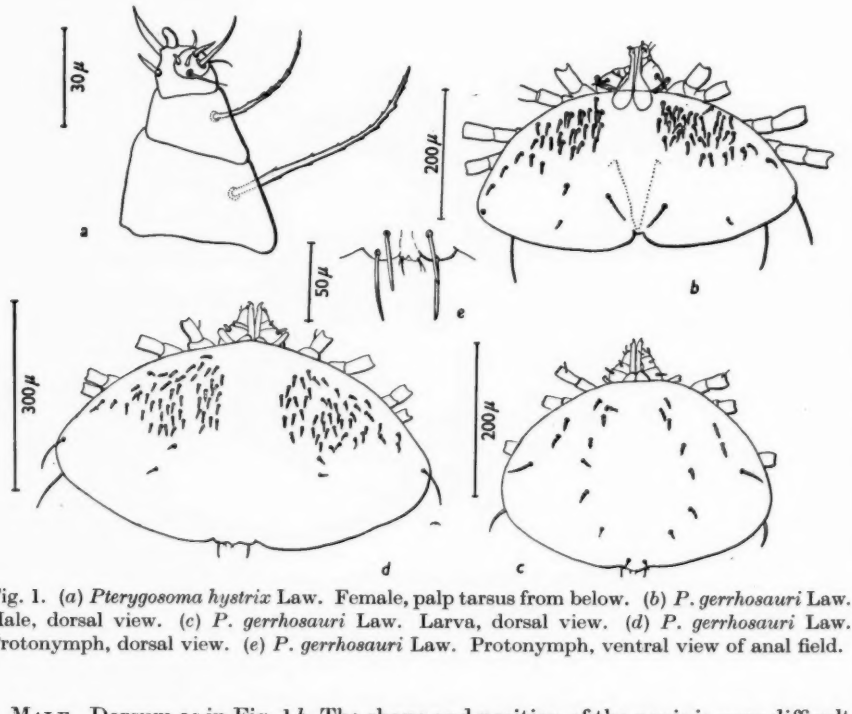

Fig. 1. (*a*) *Pterygosoma hystrix* Law. Female, palp tarsus from below. (*b*) *P. gerrhosauri* Law. Male, dorsal view. (*c*) *P. gerrhosauri* Law. Larva, dorsal view. (*d*) *P. gerrhosauri* Law. Protonymph, dorsal view. (*e*) *P. gerrhosauri* Law. Protonymph, ventral view of anal field.

MALE. Dorsum as in Fig. 1 *b*. The shape and position of the penis is very difficult to discern. The most lateral pair of peripheral setae is inserted dorsally, the other pair ventrally. The venter has three pairs of simple, inconspicuous setae. One pair at each inner corner of the posterior genital indentation of the body, a second pair placed laterally, and somewhat anteriorly, to these and a third pair, much more anterior in position, inserted nearer to the mid-line. Coxal plates with the usual setation. Hypostome, chela, and setation of the palpi as in the female.

LARVA. The dorsum as in Fig. 1 *c*. The two posterior-most pairs of setae on the dorsum are smooth, the other dorsal setae are weakly, or obviously, serrated.

There is a longer, smooth seta inserted dorsally, a little way in from the lateral margin of the body, on each side. The setation of the gnathosoma is as in the female, except that the simple ventral seta on palp segment 1 is missing.

Anal field with one pair of short, simple, dorsal setae and one pair of very slightly longer, ventral setae near the posterior margin of the anal lobe. Three pairs of short anal spines; one pair dorsally, one pair terminally, and one pair ventrally inserted.

The venter is characterized by the absence of several setae present in subsequent developmental stages. The anterior common coxa has only two setae, the seta near to the insertion of trochanter II being absent. The posterior coxa is without setae, unless a moderately long, very slender, smooth seta situated some distance from the coxa, towards the mid-line, represents the basal coxal seta. Except for these last-mentioned setae, there is no other seta on the ventral body surface.

PROTONYMPH. Dorsum as in Fig. 1*d*. Venter with three pairs of clearly visible setae on the ventral body surface, the anteriormost pair being much finer than the more posterior setae. Anal field ventrally (Fig. 1*e*) with two pairs of stout, blunt setae, somewhat longer than the other ventral setae and with one pair of anal spines inserted terminally and a second pair dorsally, near the posterior margin of the anal lobes. Coxae with the usual setation, i.e. three setae on the anterior common coxa and two setae on the posterior common coxa. Palp segment I with an elongate, slender seta inserted mid-ventrally.

DEUTONYMPH. Very similar to the female, but differing in body proportions; the legs, gnathosoma, and peripheral setae appearing disproportionately large.

The setation of the dorsum is as in the female. The intermediate-sized, mid-dorsal setae total one pair, and are usually about twice the length of the setae of the anterior dorsal patches. Occasionally, however, this pair of setae is very short, but always quite smooth, thus differing from the serrated anterior dorsal setae. One pair of dorsal anal field setae is smaller and thinner than the eight pairs of ventral anal setae. Three pairs of dorsally inserted, elongate, curved anal spines are clearly visible. A fourth pair is terminal in position, though the position is obviously dependent on the orientation of the specimen. Three pairs of elongate peripheral setae, subequal in length to those of the adult female. Mouthparts as in the female, the dorsally inserted seta, or spine, on palp segment II is very marked, being stout, serrated, and about as long as the breadth of the palp at this level.

Venter. Three pairs of setae on the ventral body surface. Eight pairs of ventral anal setae, sometimes one pair of ventral anal spines. Coxae with the usual setation for the genus, as in the female.

Material of *Pterygosoma gerrhosauri* examined: South African Mus. B. 8172, off *Gerrhosaurus validus*, Zululand; 1 ♀, 1 ♂, 5 deutonymphae, 6 protonymphae, 1 larva. S. Afr. Mus. B. 8384 off *G. validus*, Plumtree, Rhodesia; 3 ♀. S. Afr. Mus. B. 8673, off *G. validus* (locality unknown to me); 3 ♀, 1 ♂. S. Afr. Mus. B. 8725, off *G. validus* (locality unknown to me); 1 ♀, 2 ♂. 1 p. nymph. S. Afr. Mus. B. 8672, off *G. grandis*, Leydsdorp, Transvaal; 2 ♀.

Dimensions for the known stages of Pterygosoma gerrhosauri *Law*, 1935

	Character	No.	Range (μ)	Mean (μ)	Standard deviation	Coefficient of variation
FEMALE	Length	10	410–551	479	48·9	10·2
	Breadth	10	842–1073	956	76·3	8·0
	Gnathosoma length	10	127–165	148	16·2	10·9
MALE	Length	4	219–244	231	10·3	4·4
	Breadth	4	425–482	461	25·1	5·4
	Gnathosoma length	4	96–106	100	4·2	4·2
DEUTONYMPH	Length	5	308–405	347	38·9	11·2
	Breadth	5	573–719	635	59·9	9·4
	Gnathosoma length	5	136–164	156	11·4	7·3
PROTONYMPH	Length	5	251–333	297	32·4	10·9
	Breadth	5	494–591	555	40·7	7·3
	Gnathosoma length	4	87–107	99·5	7·5	7·5
LARVA	Length	1	—	230	—	—
	Breadth	1	—	352	—	—
	Gnathosoma length	1	—	74	—	—

Pterygosoma bicolor Lawrence 1935

This species appears to be very variable and some specimens bear a superficial resemblance to *P. gerrhosauri*.

FEMALE. Fig. 2 shows dorsal and ventral views of two specimens, drawn to the same scale, from opposite extremes of the series.

It will be noticed that measurements of length, breadth and numbers of intermediate-sized simple dorsal setae are very variable, as are the numbers of peripheral and anal setae, and anal spines. The latter vary from three to five, the more usual number being four, arranged as follows: a pair of moderately long, slender spines ventrally; beyond these, in a terminal position, a pair of longer, stouter, curved spines which are almost as long as the anal setae and dorsally two pairs of shorter, stout spines.

In all specimens so far examined, including the type material, there is a pair of fine, simple setae, mid-ventral in position and close to the mid-line. These setae were not mentioned by Lawrence. The setation of the palp in all specimens is in accord with the type description, i.e. segment II with a long, slender dorsal spine, weakly serrated apically (in some of the present material this spine is serrated over the greater part of its length, and varies in thickness); segment III with a stout, triangular spine, and segment IV with a stout spine mesially below the claw (in some specimens this spine tapers gradually, in others the base is stout and tapers suddenly to a whip-like distal half).

The following features distinguish *P. bicolor* from *gerrhosauri*. There are eight pairs of ventral anal setae in *gerrhosauri*, whereas in *bicolor* the maximum number of ventral anal setae recorded is six pairs. The single pair of dorsal anal field setae in *gerrhosauri* are finer and shorter than the ventral anal setae. This is not so in

bicolor. The peripheral setae of *gerrhosauri* are longer than in *bicolor*. Finally, one pair of leg setae in *bicolor* are '. . . very weakly serrate'.

Analysis shows that there is no significant difference between the variant British Museum specimens and the type material.

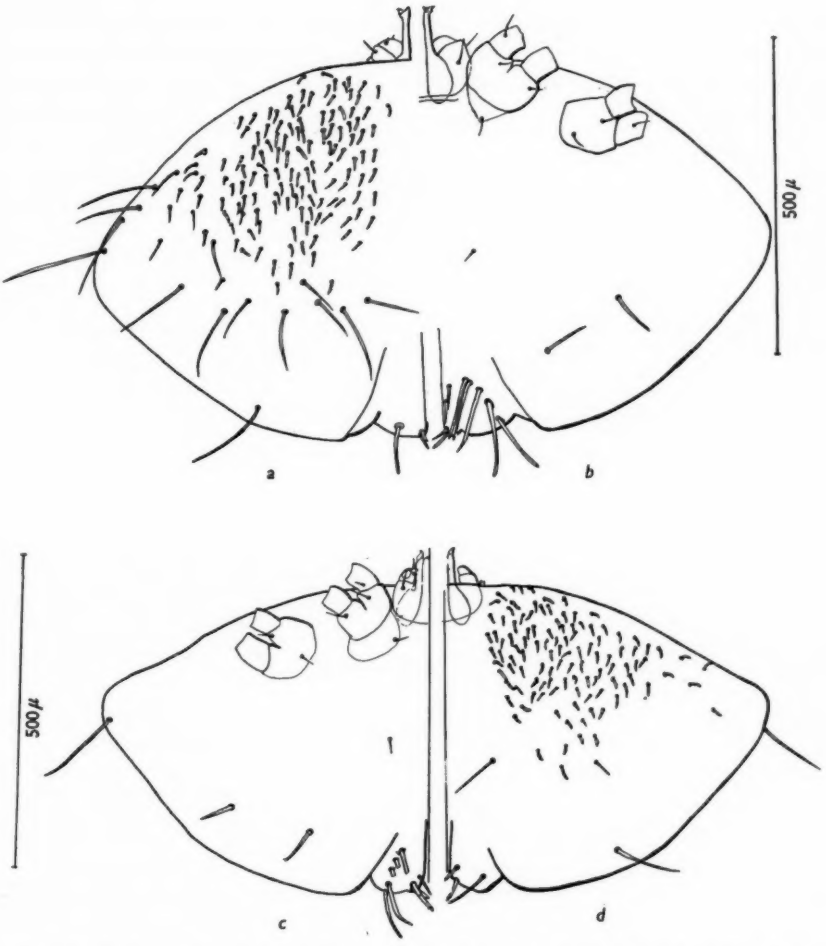

Fig. 2. *P. bicolor* Law. Female, variation in the chaetotaxy of the dorsal and ventral surfaces. (*a*) and (*d*), dorsal; (*b*) and (*c*), ventral.

Dimensions. S. Afr. Mus. B. 8387. Type material—length: 374–602 μ, mean 435 μ (40 specs.); breadth: 772–1053 μ, mean 876 μ (40 specs.); ratio, B/L: 1·65–2·16, mean 1·96; ratio gnathosoma length/body length: 3·04–4·51, mean 3·74 (36 specs.); number of intermediate-sized dorsal setae 11–29, mean 22.

Other specimens varied thus—Length: 344–622 μ; breadth: 700–1077 μ; B/L: 1·66–2·33; number of intermediate-sized dorsal setae: 2–30.

MALE. A single example of a male has been seen in the South African Museum material. This possessed three pairs of ventral setae.

Among the material from the South African Museum are two protonymphs and a deutonymph, hitherto undescribed.

PROTONYMPH. Dorsum as in Fig. 3*a*. The anal field is undeveloped, there being only two fairly short anal field setae inserted ventrally. Between these are two slightly smaller and finer anal spines on the anal lobes. Many of the longer, smooth intermediate dorsal setae are absent, while the anterior, small dorsal setae are hardly, if at all, serrate.

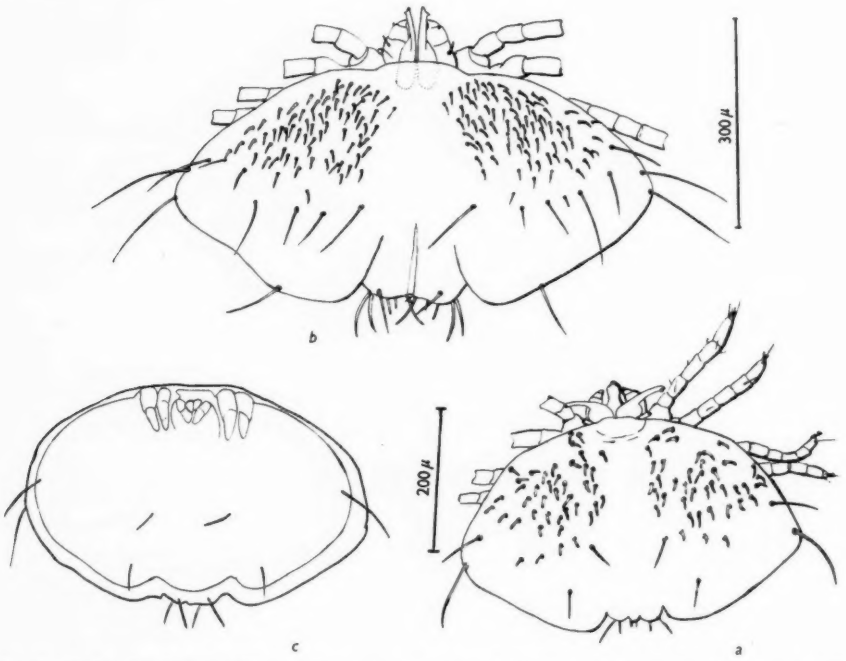

Fig. 3. *P. bicolor* Law, 1935. (*a*) Protonymph, dorsal view. (*b*) Deutonymph, dorsal view. (*c*) Nymphochrysalis, dorsal view.

The venter is equipped with three pairs of fine inconspicuous setae. The coxal plates with the usual number of setae situated as in the adult. Details of the palpi and mouthparts are very like those of the female.

A second specimen taken from the same host differs slightly from the above description in having no elongate intermediate-sized dorsal setae, and in having a single pair of simple anal field setae inserted dorsally, in addition to the two pairs of ventrally inserted anal field setae.

DIMENSIONS. Length: 294μ; breadth: 481μ; body length/chelicera length: 5·7.

DEUTONYMPH (Fig. 3*b*). General facies as in the female, but the setae, mouthparts and legs appear disproportionately large. The chelicerae are as in the female, the chaetotaxy of the dorsum is shown in Fig. 3*b*. The setation of the anal region

comprises: one pair of dorsal anal setae, slightly shorter than the other anal field setae, placed nearer to the margin of the anal lobe than it is in the female; and one pair of anal setae terminal in position; four pairs of ventrally inserted anal setae; one pair of short dorsally placed anal spines and one pair of ventral, curved, elongate anal spines.

The coxal plates with the usual setae, as in the female. Ventral body surface with three pairs of simple setae.

DIMENSIONS. Length: 332μ; breadth: 685μ; chelicerae: 120μ; L/C (body length/chelicera length): 2·77. A second unexpanded specimen measured—length: 282μ; breadth: 496μ; L/C: 2·94.

A single nymphochrysalis has also been observed (Fig. 3*c*), the details of the contained stage were difficult to discern.

HOST AND LOCALITY RECORDS

Material from the South African Museum:

B. 8387. *Gerrhosaurus flavivulgaris*, Serowe, Bechuanaland; 40 ♀, 2 deutonymphae.

B. 7810. *G. flavivulgaris*, locality unknown to me; 3 ♀.

B. 7809. *G. nigrolineatus*, locality unknown; 2 ♀.

B. 8671. *G. nigrolineatus*, locality unknown; 1 ♀.

B. 7808. *G. auritus*, Kaokoveld, S.W. Africa; 6 ♀, 2 deutonymphae, 1 protonymph, 1 nymphochrysalis.

Material from British Museum:

G. flavivulgaris, ref. no. 93:10:26:10–11, Zomba, Nyasaland; 8 ♀.

G. nigrolineatus, ref. no. 1936:8:1:471–2, Sissekab, S.W. Africa; 1 ♀.

G. nigrolineatus, ref. no. 1936:8:1:478–83, Issimbo, Amboin, Angola; 1 ♀.

G. nigrolineatus, ref. no. V.10:45:2:20, 'South Africa'; 5 ♀.

G. nigrolineatus, ref. no. 1932:9:9:80, Chundwaponde, N. Rhodesia; 1 ♀.

G. nigrolineatus, no ref. no., Parangigo do Auriaposec. 3 ♀.

SUMMARY

1. A short note is given on *Pterygosoma hystrix* Lawrence, 1935, amplifying the original description and extending the known range of variation.

2. The hitherto undescribed male, larva, protonymph and deutonymph of *Pterygosoma gerrhosauri* Law, 1935, are described and figured.

3. The protonymph and deutonymph of *Pterygosoma bicolor* Law, 1935, are described and figured, together with a record of variation in the female.

REFERENCE

LAWRENCE R. F. (1935). Prostigmatic mites of South African lizards. *Parasitology* **27**, 1–45.

(*MS. received for publication* 13. III. 1957.—Ed.)

ON THE EGG AND MIRACIDIUM OF *PARYPHOSTOMUM MEHRAI* (FARUQUI)

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(With 3 Figures in the Text)

INTRODUCTION

This paper gives an account of the mature eggs and the miracidium of *Paryphostomum mehrai* obtained from experimentally infected white rats, which were fed with the cysts of *Cercaria mehrai* obtained from naturally infected as well as artificially infected snails of the species *Indoplanorbis exustus*. The metacercariae develop into the adults in the intestine of the host in a period of 20 days, after which the adults begin to lay their eggs, which are passed out with the faeces of the host. The molluscan phase of the life cycle involves the gradual development of the miracidium larva after its penetration into the molluscan host into a mother rediae. The mother rediae, not more than three to five in number, were observed in the preoesophageal region of the snail host. They produce large numbers of daughter rediae, which were found migrating towards the liver mass. The daughter rediae, after fixing themselves in the liver mass, undergo further development and produce large numbers of cercariae. The entire process, from the entry of the miracidium until the cercariae are matured takes about 8 weeks.

THE EGG

The eggs, when they are passed out with the faeces, are entirely undeveloped. They are oval in shape, yellow and sometimes light green in colour and have a thin, transparent shell. The ovum is located in the middle of the egg and is surrounded by yolk cells.

Eggs collected in faeces, washed and allowed to stand, hatch in a week when the water temperature is about 26° C., but during winter, eggs take a longer time, even a month, to hatch and sometimes they do not hatch at all. An active miracidium may be seen within the egg much earlier, because the flame cells and cilia appear before the embryo is fully developed. At about 12–13 days, the young miracidium is somewhat spherical, and is ciliated, and has two flame cells in the body; but during the next few days, it becomes more elongated, the epidermal plates become evident, and the right flame cell shifts to a more anterior position.

MIRACIDIUM

The free-swiming miracidium larva, devoid of eye spots, is somewhat pyriform in shape, but it can change its form greatly by contraction and extension of its body. The miracidium killed with heat and vital stains has an average length of 0·017 mm. and an average width of 0·037 mm.

Locomotion

After hatching, the miracidium swims rapidly by means of its cilia, apparently in search of a host. The miracidium usually swims forward, but it can reverse its direction. Ordinarily it swims in a spiral way while revolving on its long axis. The miracidia have also been observed swimming near the surface of water and, when they hatch in large numbers, they occupy all depths from the surface to the bottom and only dead and dying miracidia remain on or near the bottom.

Epithelium

The entire outer surface of the miracidium (Fig. 2) is completely covered with cilia, which arise from 18 flattened epidermal cells. These large cells or plates are arranged in four circumferential rows, but those on the dorsal and ventral sides of the same row do not completely overlap one another. The four rows contain, from the anterior to the posterior end, 6, 6, 4, and 2 plates respectively.

Subepithelium

Beneath the ciliated epidermal plates is a subepithelial layer of long flattened cells with small nuclei which cover the entire body. This layer, as Barlow (1925) suggested in his description of the miracidium of *Fasciolopsis buski*, becomes the body wall of the mother redia.

Primitive gut

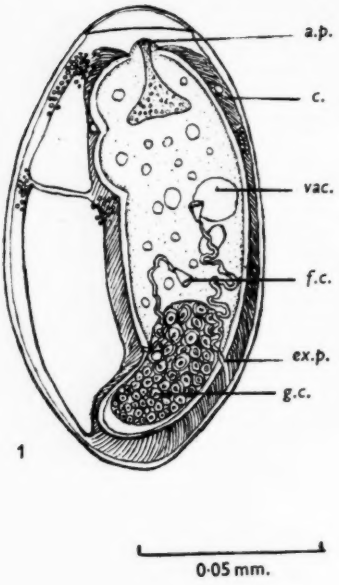
There is a sac-like structure (Fig. 1), the so-called primitive gut, which is filled with coarse granules and extends posteriorly along the axis of the body from the anterior papilla. The anterior end of the gut is situated in the centre of the apical papilla without a true oral opening and the contents of the gut apparently have no special function during the free swimming life of the miracidium. The primitive gut has no cellular structure, nor do any distinct nuclei appear to be present in it.

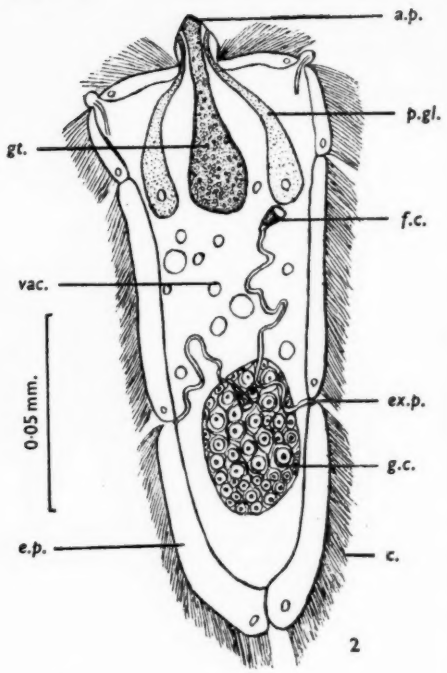
Penetration glands

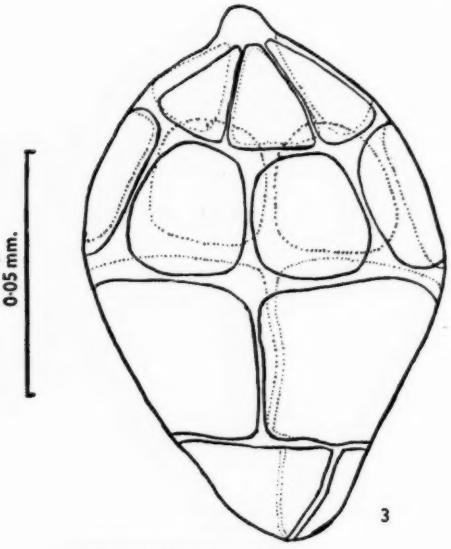
A pair of penetration glands, situated one on each side of the gut, open by their long narrow ducts a little below the tip of the papilla. Each gland cell has in its hinder region a distinct nucleus with a nucleolus and is filled with small globules of a liquid nature. There is no doubt that in structure and appearance these glands somewhat resemble the penetration glands of the forked-tailed cercariae.

Lateral processes

The miracidium here described has a pair of knob-like lateral processes between the first and second tiers of epidermal plates. It is difficult to make out any internal structures connected with the processes, but sometimes there appeared to be a duct extending inwardly and posteriorly from each process. These ducts seemed to terminate in a slightly enlarged vesicle in the middle of the second tier of epidermal cells. Although no opening of these ducts to the exterior could be noticed, a dense viscid substance was seen on several occasions to exude from each of them. They are more conspicuous when the miracidium is in a dorso-ventral







Paryphostomum mehrai

Fig. 1. Mature egg containing the miracidium.

Fig. 2. Miracidium, showing the arrangement of the epidermal plates.

Fig. 3. Miracidium showing details of its structure.

a.p., apical papilla; *c.*, cilia; *ex.p.*, excretory pore; *e.p.*, epidermal plates; *f.c.*, flame cell; *g.c.*, germ cell; *gt.*, primitive gut; *p.gl.*, penetration gland; *vac.*, vacuole.

position, but they are small or disappear from view as the position changes. Because the ducts leading from the lateral processes were not traced inwards into gland cells, the term lateral secretory glands used for them by Price (1931), and by Faust & Hoffman (1933) in their descriptions of the miracidia of *Schistosomatium douthitti* and *Schistosoma mansonii* respectively cannot be used. The secretions already referred to as being associated with these ducts might indicate the presence of these glands.

The lateral papillae and eye-spots are not present in the miracidium.

Excretory system

The excretory system of the miracidium is similar to that described by Beaver (1937) in the miracidium of *Echinostoma revolutum*. It consists of only one pair of flame cells. The right flame cell is situated at about the posterior level of the penetration glands and the left flame cell a little behind this, at about the middle of the third tier of epidermal plates. The ducts of the flame cell are much convoluted. Each collecting duct before opening to the exterior enlarges at its terminal end to form a vesicle. The excretory pores lie on each side between the third and fourth tiers of epidermal plates. The flame cell formulae is 2 (1).

Germ cells

The germ cells lie in the germinal sac and occupy the posterior two-thirds of the body. They are relatively large and contain a nucleus with a conspicuous nucleolus. They do not lie freely in the cavity of the germinal sac, but are attached to the wall by fibre-like extrusions of the cells.

DISCUSSION

Echinostome miracidia which have been described in detail are those of *Echinoparyphium recurvatum* Rasin, 1933 and *Echinostoma revolutum* Beaver, 1937. The miracidium of the present species agrees in general morphology with those of the above two species. The cuticular plates are arranged in numbers of 6, 6, 4 and 2. A similar condition was observed in the miracidium of *Spirorchis parvus* (Wall, 1941).

The excretory system of the miracidium of *Paryphostomum mehrai* (Faruqui, 1930) is practically similar to that of *Echinostoma revolutum* (Beaver, 1937). The two flame cells with their convoluted ducts empty between the third and last row of plates, but eye-spots are absent from the miracidium of the species here described and the germ-balls lie in a germinal sac, whilst in the miracidian *Echinostoma revolutum* and *Echinoparyphium recurvatum* eye-spots are present and the germ-balls lie loose in the posterior two-thirds of the body. The number and arrangement of the germ-balls are variable.

It gives me great pleasure to express my indebtedness to Dr H. R. Mehra, under whom this work was conducted, for his valuable guidance. I also wish to express my sincere thanks to the Uttar Pradesh Government Scientific Research Committee for the grant of a Research Assistantship under Dr H. R. Mehra for this work.

REFERENCES

BARLOW, C. H. (1925). The life cycle of the human intestinal fluke *Fasciolopsis buski* (Lankester). *Amer. J. Hyg. Monogr. Ser.* no. 4, 1–98.

BEAVER, P. C. (1937). Experimental studies on *Echinostoma revolutum* (Froelich), a fluke from birds and mammals. *Illinois Biol. Monogr.* **15**, 1–96.

FARUQUI, A. B. (1930). On a new species of Cercariae indicae found in *Indoplanorbis exustus* (Deshayes). *Indian J. Med. Res.* **17**, 1205–14.

FAUST, E. C. & HOFFMAN, W. A. (1933). Studies on *Schistosomiasis mansoni* in Puerto Rico. III. Biological studies. I. The extra-mammalian phases of the life cycle. *Puerto Rico J. publ. Hlth*, **10**, 1–47.

PRICE, E. W. (1931). Life history of *Schistosomatium douthitti* (Cort). *Amer. J. Hyg.* **13**, 685–727.

RASIN, K. (1933). *Echinoparyphium recurvatum* (Linstow, 1873) und seine Entwicklung. [German summary.] *Biol. Spisy. Vysoké Skloy Zvěrolék*, Brno, **12**, Art. 1–2, 1–98.

WALL, L. D. (1941). *Spirorchis paruus* (Stunkard), its life history and the development of its excretory system (Trematoda: Spirorchiidae). *Trans. Amer. Micro. Soc.* **60**, no. 2, 221–60.

(*MS. received for publication* 3. IV. 1957.—Ed.)

EXPERIMENTAL *EIMERIA MITIS* INFECTIONS IN CHICKENS

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(With 6 Figures in the Text)

I. INTRODUCTION

In the past, research on the coccidia of the domestic fowl has been largely concentrated on *Eimeria tenella*, which has always been an important cause of losses among young chicks. This species of *Eimeria* parasitizes the caeca, but other species which invade intestinal tissues have recently also assumed importance. This is because modern methods of intensive husbandry have increased the risks of exposure to heavy infestations with the result that several species which formerly were associated with only benign conditions have been identified as the cause of losses.

As part of a programme of research on those species of *Eimeria* which parasitize the intestinal tissues of domestic fowls, attempts have been made to isolate the species found in poultry deep-litter houses in order to investigate their pathogenic properties and to devise methods of control. Each species appears to require different conditions, and it has proved necessary to develop special methods for their isolation and maintenance in the laboratory. It was in the course of this work that a strain of *E. mitis* was isolated and, as very little attention has been devoted to this species since its first brief description by Tyzzer (1929), the opportunity was taken of studying some of its characteristics in greater detail.

II. MATERIALS AND METHODS

Isolation of single oocysts

The micropipette used for the isolation of single oocysts was designed and constructed in the Laboratory workshop. The essential details of its construction are shown in Fig. 1.

In operation, the cylinder is first filled with oil from the reservoir by connecting the reservoir (*B*) with cylinder (*A*) via the tap (*C*) and withdrawing the piston. The tap (*C*) is then turned to connect the cylinder with the polythene delivery tube. The oil is ejected into the tube and the pipette filled completely with the oil. The instrument is then ready for use.

By attaching a lever (*E*) at right-angles to the piston-controlling shaft more delicate control of the meniscus at the tip of the pipette can be obtained. The pipette (*F*) is drawn out from 2 mm. glass tubing, the bore at the tip being approximately 100μ. It is held at the tip of a lever (*D*) which is mounted in a universal joint on the top of the Perspex block as shown. The length of the lever (*D*) from

the joint is $8\frac{1}{2}$ in. and the distance from the joint to the tip of the pipette is 3 in. The height of the Perspex block can be adjusted and it can also be rotated about the axis of the supporting column.

The manipulations are carried out under the microscope using a $\frac{2}{3}$ in. objective and a 10× eyepiece. A drop of a suspension of oocysts is placed on a slide and brought into the field of focus. By means of the lever (*D*) the pipette is manœuvred until the tip is immersed in the drop of fluid and also in the field of focus. A few drops of oil are ejected from the pipette to ensure that the system is completely

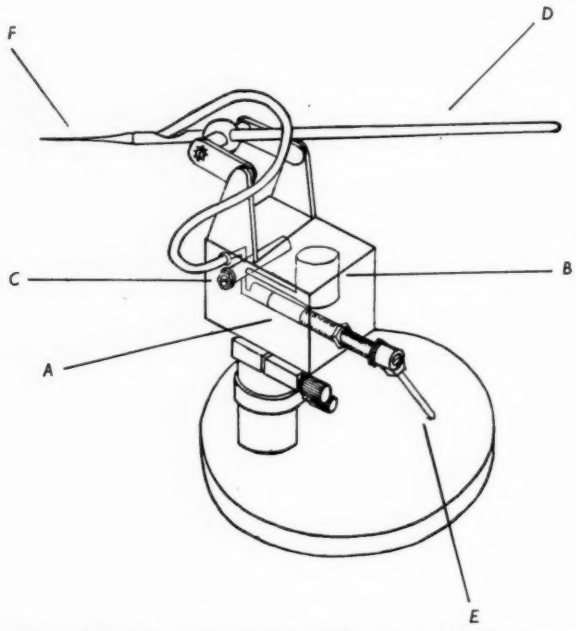

Fig. 1. The micropipette used for the isolation of single oocysts. The apparatus consists of a Perspex block measuring $1\frac{1}{2} \times 1\frac{3}{4} \times 2$ in. drilled to accommodate the following: *A*, a cylinder within which a piston moves controlled by a screw shaft, the pitch of the thread on the shaft was 40 per inch and the bore of the cylinder was 6·75 mm.; *B*, a reservoir containing about 5 ml. of a low-viscosity oil (Icematic, Wakefield); *C*, a three-way tap by means of which the cylinder (*A*) could be connected in turn with the reservoir (*B*) or via a polythene tube to the pipette, *F*. The levers (*D*) and (*E*) respectively control the pipette (*F*) and the piston (*A*).

full of oil. The pipette remains fixed and the slide is moved on the mechanical stage until a suitable oocyst is close to the tip of the pipette. Slight withdrawal of the plunger causes the oocyst to be drawn into the pipette. The pipette is then raised out of the liquid and a small piece of agar is introduced on the slide into the microscope field. The pipette is lowered and the drop of liquid containing the oocyst is expelled on to the agar. The oocyst is measured with the aid of an eyepiece micrometer and the agar is transferred with forceps on to the tongue of a young chick.

Maintenance of chicks

Rhode Island Red or White Leghorn chicks were reared in isolation from adventitious coccidial infection. As an added safeguard before the birds were subjected to experiment, samples of their droppings were examined to ensure the absence of oocysts.

At first the chicks were housed in small metal cages completely enveloped in muslin which was soaked daily in ammonia solution, but the later experiments were carried out in an isolation room with a ventilation system independent of other rooms, where experiments with other species of *Eimeria* were in progress. This room was entered through double doors and before entering the inner room the attendants changed their overalls and washed their boots.

Handling of oocysts

Oocysts were separated from droppings by flotation in saturated salt solution. The droppings were collected from the trays beneath the cages and suspended in 2% potassium bichromate solution. The suspension was strained through muslin and spun in a centrifuge for 10 min. at approximately 2000 r.p.m. The deposit was resuspended in saturated salt solution and again centrifuged. The upper layers of the supernatant fluid, which contained the majority of oocysts free from debris, were transferred to 1 l. cylinders of water and allowed to sediment overnight. The oocysts were recovered from the deposit and resuspended in 2% potassium bichromate, where they were allowed to sporulate at room temperature in shallow dishes and finally stored at +5° C.

Oocysts separated and stored by this method appeared to deteriorate after about 4–6 weeks, when the numbers of cracked oocysts progressively increased. Cultures of oocysts therefore were never used for experiments more than 1 month after preparation.

The oocysts in droppings were enumerated by shaking 3 g. samples with glass balls in 42 ml. of water. The mixture was strained through muslin, and of the resulting filtrate 15 ml. were spun in a centrifuge at 1500 r.p.m. for 3 min. The supernatant fluid was discarded and the sediment resuspended in saturated salt solution. The numbers of oocysts in a sample of this suspension were counted on a McMaster Slide.

Isolation of a strain of Eimeria mitis

The strain of *E. mitis* used in these experiments was isolated from a suspension of oocysts separated from a sample of litter obtained from a broiler house in Hampshire. These oocysts were separated from the litter by a flotation process similar to that described above for droppings.

In all, twenty chicks were infected with single oocysts obtained by the method described above. The measurements of the selected oocysts lay within the limits of 14–20 × 12–16 μ. Infections developed in thirteen of these chicks. The strain finally used in all subsequent experiments was derived from the gut content of a chick 6 days after infection.

III. RESULTS

Characteristics of the oocysts and oocyst production

In all the experiments a regular examination of the droppings was made during the first 7 days after infection. This was one of the precautions taken to avoid adventitious infections. The first oocysts always appeared during the 5th day after infection. An attempt was made to determine the prepatent period more precisely by infecting separate groups of chicks at three different times during the day from the same culture. During the 5th day after infection these three groups contained chicks covering periods of infection from 96 to 116 hr. Oocysts were first detected in small numbers 101 hr. after infection.

Sporulation was followed in a constant temperature cabinet at 60° F. (15·5° C.) at which temperature formation of sporozoites was detected at 48 hr. after separation of the oocysts from intestinal contents and 25% of the oocysts were completely sporulated at 64 hr. In the course of the preparation of suspensions of oocysts for experiments, sporulation at ordinary room temperatures was always well advanced 48 hr. after collection.

The general morphological characteristics of the oocysts closely agreed with the description given by Tyzzer (1929), although the oocysts were slightly smaller in size. Measurements of 100 oocysts followed unimodal distributions with a mean length of 15·58μ and breadth of 13·83μ, within the ranges of 11·5–20·7μ and 10·35 and 18·4μ.

The oocysts were subspherical, being only slightly tapered at one end; the enclosed zygote was approximately spherical and almost completely filled the lumen of the oocyst; a conspicuous refractile body was also commonly present.

In the experiments recorded in Fig. 2*a* and *b* an attempt was made to assess the production of oocysts following a known infection. Two identical groups of twelve chicks aged 3 weeks were kept in wire-floored metal cages in the isolation room. The cages were enclosed in muslin and the droppings were removed daily. One group of chicks received 100,000 oocysts and the other received 1000 oocysts. The total daily production of droppings from each group was mixed and weighed. Duplicate samples of 3 g. were removed and the oocyst content was determined. The total daily oocyst output was thus estimated.

The results recorded in Fig. 2*a* and *b* show that in both groups the first oocysts appeared during the 5th day after infection. The production of oocysts by chicks receiving the smaller inoculum ceased during the 14th day after infection and in the group receiving the larger dose, oocyst production ceased during the 15th day. The total numbers of oocysts recovered were $61{\cdot}709 \times 10^6$ from chicks receiving 1000 oocysts and $225{\cdot}343 \times 10^6$ from those receiving 100,000 oocysts.

In both groups of chicks, oocyst production ceased abruptly. There did not appear to be a gradual diminution in the numbers of oocysts, but in the group receiving the larger inoculum, oocyst production was erratic during the final 3 days of the infection. Oocyst production in both groups took place during approximately the same period, i.e. 5–15 days after infection, but the total number of oocysts produced by chicks receiving the larger inoculum was only 3·65 times

more than the number produced by the other group, although they received 100 times more oocysts at infection.

This is in agreement with the observations of Brackett & Bliznick (1952) who concluded from their observations on some other species of *Eimeria* that there is no constant relationship between the size of the infective dose and the number of oocysts produced.

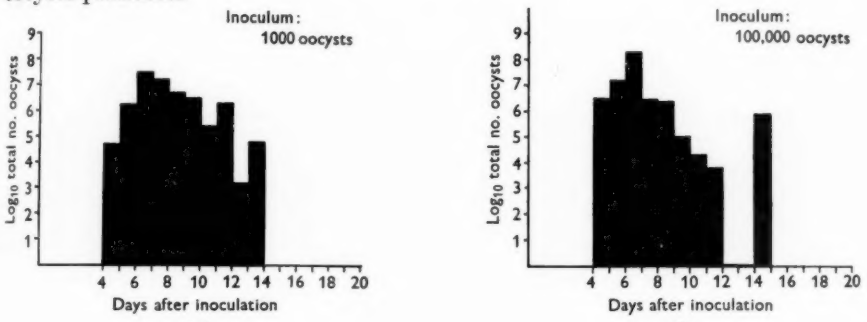

Fig. 2*a*, *b*. The production of oocysts following infection with (*a*) 1000 oocysts, (*b*) 100,000 oocysts.

Pathogenicity

E. mitis has never been associated with serious disease, but the report of Allen (1934) and the opinion of Levine (quoted by Brackett & Bliznick, 1950) suggest that this species may not be entirely non-pathogenic. This is confirmed by the experiments recorded in Table 1, where chicks of different ages were given varying doses of oocysts.

In every case the growth of the chicks receiving the larger doses of oocysts was markedly retarded. In Expts. 1 and 2A (Table 1), where chicks aged 6 days received either 2·5 millions or 500,000 oocysts, weight gains were seriously reduced by the 4th day after infection. With older chicks receiving similar doses (Expts. 2B

Table 1. *The effect of* Eimeria mitis *infections on the growth of chicks of different ages*

Expt. no.	Age of chicks (days)	No. of oocysts	No. of chicks per group	No. of deaths	Average body weight (gm.)		
					Initial	4th day	8th day
1	6	0	30	0	51·6	70·3	88·0
		10,000	30	0	53·3	64·1	83·6
		2,500,000	29	11	51·0	50·5	59·5
2A	6	0	15	0	62·2	84·9	113·4
		10,000	15	0	59·9	79·4	102·5
		500,000	14	1	58·6	60·38	69·9
2B	17	0	15	0	151·2	196·3	237·7
		10,000	15	0	151·2	196·3	226·8
		500,000	15	0	143·3	162·3	185·0
3	26	0	20	0	345·0	408·2	459·2
		10,000	20	0	359·0	427·0	452·3
		1,000,000	20	0	350·5	399·0	430·4

and 3, Table 1) the inhibitory effects on growth were apparent by the 8th day, while with smaller doses of oocysts it was only the younger birds which were affected. In the older chicks aged 17 or 26 days (Expts. 2B or 3, Table 1), which received 10,000 oocysts, there was no significant effect.

The only deaths occurred in the youngest chicks which received the heaviest infections. In birds which died 6–9 days after infection, there was no evidence of haemorrhage nor were any gross lesions detected.

In order to observe these growth-retarding effects in greater detail the growth of three groups of ten 6-day-old chicks was recorded graphically in Fig. 3. The average weight of a group of normal uninfected chicks was compared with two groups of similar chicks which received 10,000 and 500,000 oocysts respectively.

In the group which received 500,000 oocysts it will be seen that growth was markedly inhibited between 2 and 7 days after infection. In the group which received only 10,000 oocysts the inhibition of growth took place more gradually over approximately the same period.

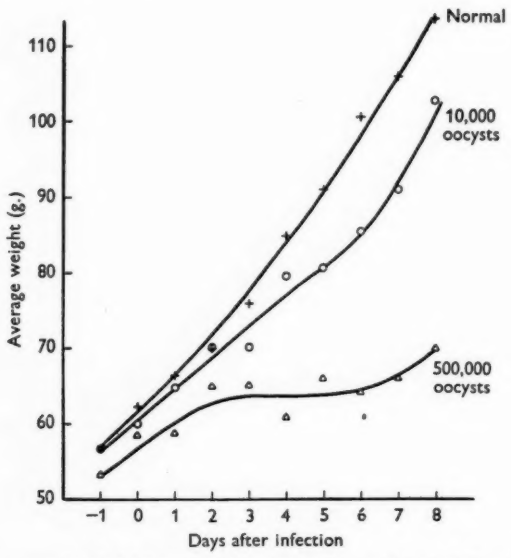

Fig. 3. The growth of chicks receiving different numbers of *E. mitis* oocysts.

Developmental stages of Eimeria mitis *in intestinal tissue*

Attempts were made to find the different developmental stages of *E. mitis* by examining intestinal tissue of chicks at varying intervals after infection with 500,000 or 1 million oocysts. Tissues were fixed in either formol saline or Bouin's solution for staining with Ehrlich's haematoxylin and eosin or Heidenhain's iron haematoxylin. An additional series of preparations was fixed in Carnoy's fixative and stained with Giemsa's stain (Shortt & Cooper, 1948).

Free merozoites were first found in intestinal contents on the 4th day after infection. In sections, however, schizonts were first demonstrated slightly before

this, i.e. 67 hr. after infection. Stages prior to schizonts were not identified with certainty: this repeats the experience of Tyzzer (1929), who also failed to find early developmental stages.

Mature schizonts were nearly always situated superficially in the epithelium (Fig. 4) and they appeared to be evenly distributed with no tendency to aggregate. Tyzzer (1929) observed that *E. mitis* developed in greatest numbers in the upper portion of the small intestine. In the present series of experiments, however, schizonts and free merozoites first occurred in regions of the intestine anterior to the yolk stalk, but they were not confined to this section of the gut. Gametocytes appeared to occur throughout the intestine, and in one series of sections they were found in greatest concentrations posterior to the yolk stalk (Fig. 5).

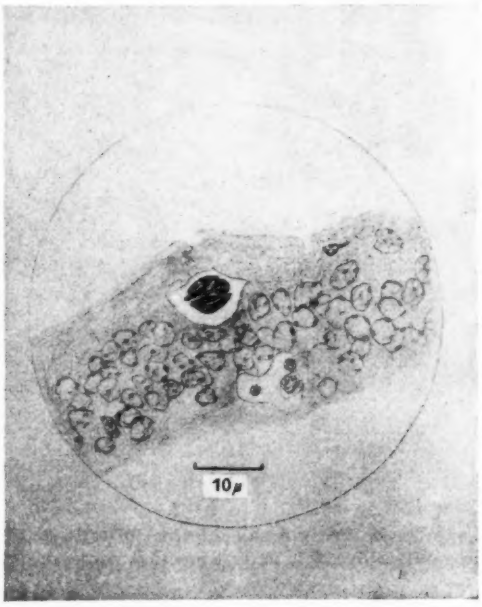

Fig. 4. Schizont of *E. mitis* in the small intestine of a chick, 100 hr. after infection.

The schizonts most commonly produced about 16 merozoites which were crescentic in shape. The structure of a typical mature schizont is shown in Fig. 4, where the characteristic polar position of the spherical residual body is seen.

Gametocytes were not seen in sections until 120 hr. after infection, although oocysts generally appeared some hours before this: this may have been because they were very scanty at first and gradually increased in numbers. In some cases they became very numerous, as seen in Fig. 5 drawn from a section of posterior intestinal tissue, 196 hr. after infection. In the developing macrogametocytes eosinophylic 'plastic granules', which have been studied recently by Patillo & Becker (1955) in *E. brunetti* and *E. acervulina*, have not so far been recorded in *E. mitis*. As seen in Fig. 5, however, structures corresponding to these granules

were present, situated at the periphery of the immature macrogametocytes and stained prominently with eosin. The microgametocytes correspond to Tyzzer's (1929) description, and the single mass of residual material which he noted can be seen in Fig. 5.

Tyzzer (1929) considered it a peculiarity of *E. mitis* that schizonts and gametocytes often occur together. This was not found to be a constant feature: in several preparations, sections of small intestine contained numerous gametocytes in the absence of any schizonts. The two stages were observed together, however, in sections of the intestine of a chick which died 120 hr. after receiving 500,000 oocysts. Several gametocytes and schizonts were present in the same microscopical field.

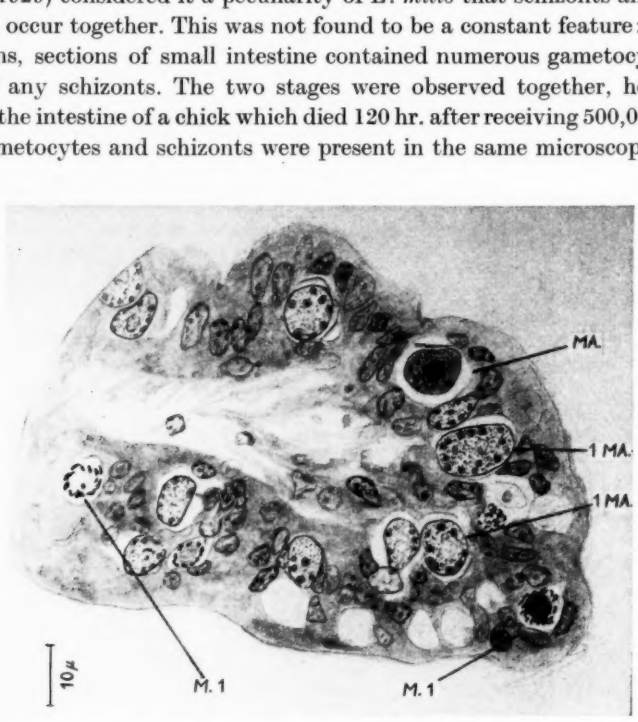

Fig. 5. Gametocytes of *E. mitis* in the small intestine of a chick 196 hr. after infection. 1 *MA.*, immature macrogametocyte; *MA.*, mature macrogametocyte; *M.* 1, microgametocyte.

Lesions which could be associated with the development of the parasites were not observed: probably the mild pathological changes which have been demonstrated are due to some effect directly dependent upon the number of tissue cells invaded, possibly as a result of the inactivation of epithelial cells by the parasites.

Control by sulphadimidine

In a species of *Eimeria* with a relatively low pathogenicity, measurable effects due to the parasites cannot always be observed regularly and experiments to demonstrate the effects of drugs or immunity are correspondingly difficult. Only two properties of *E. mitis* infections were sufficiently constant to provide criteria of the inhibitory effects of drugs. These were the reduction of the growth rate and the production of oocysts. A few experiments were carried out to determine to what extent these factors could be influenced by drug action.

One of the experiments is recorded in Fig. 6, where the weights of three groups of chicks are plotted. Group *A* were uninfected and maintained under the same conditions as groups *B* and *C* which received 500,000 oocysts of *E. mitis* on day 0. Group *B* received in addition 0·2 % sodium sulphadimidine in the drinking water for 4 days commencing at the time of infection. It will be seen that the weight of the untreated group (*C*) was consistently below that of the others after the 3rd day following infection. As an additional control a 4th group was included which, for the sake of clarity, has been omitted from Fig. 6. In this 4th group uninfected chicks were isolated in a separate room and they received 0·2 % sulphadimidine in the drinking water for the same period as group *B* (day 0 to day 4). The weights of these chicks agreed closely with those of groups *A* and *B*, the average weight on day 0 after infection being 87·3 g. (compared with 85·1 g. for group *A* and 87·3 g. for group *B*), and on the 7th day 132·4 g. (compared with 136·9 g. for group *A* and 127·6 for group *B*).

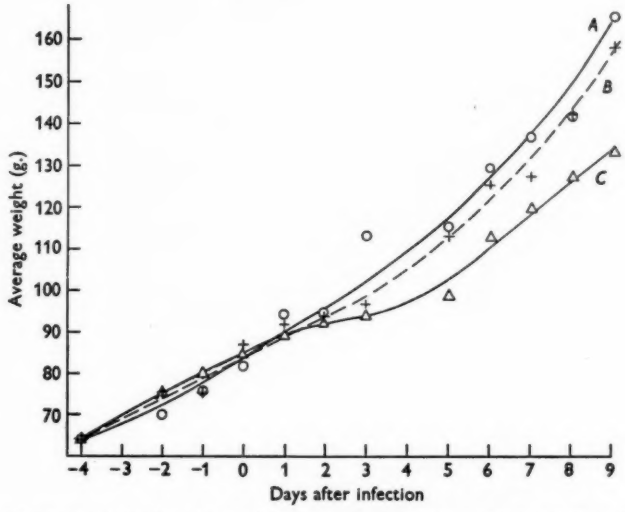

Fig. 6. The effect of sulphadimidine on the growth-retarding effects of *E. mitis*. Group *A* uninfected; groups *B* and *C* received 500,000 oocysts on day 0; group *B* received 0·2 % sodium sulphadimidine in the drinking water for 4 days commencing on day 0.

Daily examinations of the droppings were made, and in the untreated group oocysts appeared normally on the 5th day. No oocysts were recovered from group *A* during the experiment, but in group *B* the appearance of oocysts was delayed until the 5th day after removal of the drug. There was no significant retardation of growth which could be attributed to the renewed development of the parasites after withdrawal of the drug. It would be impossible to say whether this was due to the development of induced immunity or to the increasing resistance of the birds with age.

It is evident from this experiment that sulphadimidine controlled the development of *E. mitis* but did not completely eliminate the infection.

Tyzzer (1929) concluded that immunity to *E. mitis* was very slow to develop and chicks which recovered from infection with this species could be reinfected several times. This was confirmed in two experiments in which chicks were reinfected after the cessation of oocyst production following a primary infection. It was found that a further production of oocysts occurred when the first infection was suppressed with sulphadimidine as well as when it was allowed to progress normally. The effects of repeated infections on the weight of the birds could not be accurately assessed because of the small magnitude of the changes observed.

IV. DISCUSSION

The characteristics of the strain of *E. mitis* which has been isolated are in general agreement with data published by other authors. The oocyst measurements compare closely with those recently published by Becker (1956), although they are slightly smaller than those given by Tyzzer (1929) and Edgar (1955). The prepatent period of 101 hr. lies between the figures given by Tyzzer (1929) and Edgar (1955). The sporulation time agrees with that observed by Tyzzer (1929) at the same temperature. Accurate figures of developmental stages of this species have not hitherto been published, but the parasites shown in Figs. 4 and 5 correspond to the descriptions published by Tyzzer (1929).

Tyzzer (1929) suggested that the site of predilection for this species was the anterior part of the small intestine, although the parasites were not confined to this region of the gut alone. In the present study the parasites occurred throughout the intestine and in the sections examined they did not appear to predominate in the anterior part of the gut, but it is possible that the very earliest invasion of tissue occurs in the anterior intestine, and the posterior gut becomes parasitized as the infection develops. Free merozoites were first found near the duodenum, and the numbers of parasites observed in sections of intestine posterior to the yolk stalk appeared to increase during the later stages of infection.

It is evident from the data presented in this paper that, although *E. mitis* is capable of causing limited mortality in small chicks and retarding their growth, it is unlikely to be of any great pathological importance under the conditions of normal poultry husbandry. Its harmful effects were only evident when young birds received large doses of oocysts. This could take place if young chicks were suddenly turned on to heavily infested litter; but this does not occur in good husbandry practice, and in any case under these conditions other more pathogenic species such as *E. tenella* or *E. necatrix* would cause far greater losses.

Another case where *E. mitis* might cause losses was suggested by Levine (see Brackett & Bliznick, 1950), who pointed out that because *E. mitis* does not appear to stimulate readily a resistance to further infection in the chicks, it might be possible under certain circumstances for the birds to become constantly reinfected and so build up a chronic condition leading to impaired weight gains. There is little doubt that immunity to *E. mitis* develops slowly, but the build-up of infection would have to occur very rapidly before the increasing age of the birds reduced their susceptibility.

The results of the experiments with sulphadimidine suggest that this infection is amenable to chemotherapeutic control, which is also effective against other species. It would be of interest, however, to know the effects of continuous medication with low doses of preventive drugs. Levine (1943) has already shown that *E. mitis* is susceptible to low doses of sulphaguanidine given continuously which do not control some other species.

It was one of the purposes of this investigation to gain experience in techniques for isolating and handling species of intestinal coccidia. Davies (1956) isolated *E. necatrix* from infections established by the inoculation of single oocysts isolated with the apparatus described in this paper. It is agreed that the weakness of this method is that it is difficult to be certain of the identity of the oocyst selected.

The micropipette is simple in principle and operation and once adjusted, so that the tip of the pipette falls into the field of focus of the microscope, a series of isolations can be carried out without difficulty. It has also been used successfully in this laboratory for the isolation of single trichomonads in the course of culture experiments with *Trichomonas foetus*.

There were a number of special difficulties in working with *Eimeria mitis*. First, in order to obtain adequate yields of oocysts it was necessary to separate them from faeces, whereas with other species, such as *E. tenella* or *E. necatrix*, sufficient oocysts can be isolated from intestinal tissues. In this way species which invade particular regions of the gut can be separated, but with *E. mitis* this opportunity of selection was not available. It was necessary, therefore, to take precautions to isolate the infected chicks with great care to avoid adventitious infections.

The benign nature of *E. mitis* is an added difficulty in experimentation. Large inocula had to be administered to small chicks in order to obtain measurable effects. Under these conditions intercurrent infection with a relatively small dose of a more pathogenic species, which would mask the effects of *E. mitis*, could easily be introduced. This emphasises the necessity of carrying out the experiments in isolation.

The sensitivity of chicks to the clinical effects of *E. mitis* infection apparently diminishes with age. Chicks can be reinfected several times, but it is difficult to demonstrate clear effects upon growth rates when the experiments have to be prolonged beyond the period of maximum susceptibility. This short period of susceptibility is probably the main reason why *E. mitis* is not a serious pathogen under field conditions.

V. SUMMARY

1. Methods are described for the isolation and maintenance of a pure strain of *Eimeria mitis*.

2. The prepatent period of the infection in chicks was 101 hr. The mean dimensions of the oocysts were $15{\cdot}38 \times 13{\cdot}83\mu$, and sporulation occurred after 48 hr. at 60° F. (15·5° C.). The production of oocysts following infection with known numbers of oocysts is recorded.

3. When 500,000 or more oocysts of *E. mitis* were given to chicks aged 6, 17 or 26 days the growth rate of the birds was markedly reduced. Young chicks were

most susceptible and a few deaths occurred when 6-day-old chicks received 2·5 million oocysts. *E. mitis* is unlikely to be of pathological importance under normal conditions of good husbandry practice.

4. Pathological lesions due to this species of *Eimeria* were not found in sections of intestinal tissues.

5. Developmental stages of this parasite were found throughout the whole of the small intestine.

6. The growth-retarding effects and oocyst production by *E. mitis* were controlled when 0·2% sodium sulphadimidine was given in the drinking water.

Acknowledgements are due to the Staff of the Laboratory Workshop for their assistance in the design and construction of the micropipette.

REFERENCES

ALLEN, E. A. Quoted by Becker, E. R. (1934). *Coccidia and Coccidiosis of Domesticated Game and Laboratory Animals and of Man.* Monograph No. 2, Division of Industrial Science, Iowa State College.

BECKER, E. R. (1956). Apropos oocyst measurements. *J. Parasit.* **42**, (4), Suppl. 24.

BRACKETT, S. & BLIZNICK, A. (1950). *The Occurrence and Economic Importance of Coccidiosis in Chickens.* American Cyanamid Company, New York.

BRACKETT, S. & BLIZNICK, A. (1952). The reproductive potential of five species of coccidia of the chicken as demonstrated by oocyst production. *J. Parasit.* **38**, 133–9.

DAVIES, S. F. M. (1956). Intestinal coccidiosis in chickens caused by *Eimeria necatrix. Vet. Rec.* **68**, 853–6.

EDGAR, S. A. (1955). Sporulation of oocysts at specific temperatures and notes on the prepatent period of several species of avian coccidia. *J. Parasit.* **41**, 214–16.

LEVINE, P. P. (1943). The selective action of sulfaguanidine on avian coccidia. *J. Parasit.* **29**, 362–3.

PATILLO, W. H. & BECKER, E. R. (1955). Cytochemistry of *Eimeria brunetti* and *E. acervulina* of the chicken. *J. Morph.* **96**, 61–96.

SHORTT, H. E. & COOPER, W. (1948). Staining of microscopical sections containing protozoal parasites by modification of McNamara's method. *Trans. R. Soc. Trop. Med. Hyg.* **41**, 427.

TYZZER, E. E. (1929). Coccidiosis in gallinaceous birds. *Amer. J. Hyg.* **10**, 269–382.

(*MS. received for publication* 4. IV. 1957.—Ed.)

STRUCTURE, HATCHING AND SIZE VARIATION OF THE SPORES IN A SPECIES OF *NOSEMA* (MICROSPORIDIA) FOUND IN *HYALOPHORA CECROPIA* (LEPIDOPTERA)

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(With 1 Figure in the Text)

I. INTRODUCTION

No comprehensive monograph on the Microsporidia has been published since that of Kudo in 1924. The classification of the order is not satisfactory, particularly at the species level. This is due partly to the difficulties inherent in the study of such minute organisms and partly to inadequate descriptions. The identification of many species is based on the dimensions of their spores. Although it has long been known (see Kudo, 1924) that the size and shape of the spores of a single species may vary considerably, some authors give little or no indication of such variation in their descriptions. A notable exception is Blunck (1954), who measured spores of *Nosema polyvora* in different stages and different tissues of a single host species, *Aporia crataegi*, and concluded that spores vary in size (i) in different stages of the host, (ii) in the same tissue of different individuals at the same stage of development, and (iii) in different tissues. Unfortunately the number of spores measured was rather low (4–47).

There is greater variation in the size of spores in some species than in others. The greatest range recorded is in *Nosema marionis* which has spores varying from 1·5 to 7 μ in length (Stempell, 1919). The smallest microsporidian spores are those of *N. pulvis* (Perez, 1905) or possibly *N. gammari* (van Ryckeghem, 1930), which were given as $1{\cdot}25 \times 1{\cdot}0\,\mu$ and $1{\cdot}5 \times 0{\cdot}75\,\mu$ respectively.

The species of *Nosema* used in the present study was found in *Hyalophora* (*Platysamia*) *cecropia* (Lepidoptera, Saturniidae) in the laboratory of Professor Carroll M. Williams of Harvard University. Dr L. H. Finlayson, who brought the organism to this country, has given valuable advice and encouragement to the author. In this paper are presented observations on the spore, with particular reference to variation in size. Its effects on the metamorphosis of the hosts are reported elsewhere (Finlayson & Walters, 1957).

II. MATERIAL AND METHODS

Passages of the parasite were made in pupae of *Antheraea* (*Telea*) *polyphemus* and later in *A. pernyi*, and these two species were used for subsequent study of the microsporidian. The original host species has not proved suitable for laboratory rearing. The saturniids were reared at 25° C. on a diet of oak, apple or hawthorn (*A. polyphemus*) or on apple, oak, sweet chestnut, or evergreen oak (*A. pernyi*).

Host larvae are most readily infected by painting an aqueous suspension of spores on the foliage and allowing it to dry, and by starving the larvae for 12–24 hr. before feeding. For pupae it was found convenient to inject a suspension of spores in Ringer solution from a hypodermic syringe, while the animals were anaesthetized by carbon dioxide. All materials were sterilized, and crystals of phenylthiourea were added to the suspension to inhibit the action of tyrosinase which causes the blood to blacken and become toxic (Williams, 1952).

The life history has been studied by observation of fresh material, smears and sections. Giemsa staining (Shute's modification, 1955), as well as the Giemsa-colophonium method (Shortt & Cooper, 1948), has been used for sections. Fontana's silver impregnation method was used for staining the polar filaments. Electron microscopy has been used in the study of the polar filament.

Spores were measured under several conditions, and also from several different host tissues; in each case 100 were measured. This was done by means of camera lucida drawings at a magnification of $\times 1140$. Since the breadth and length of the spores vary independently, it was necessary for the purpose of comparison to calculate the volume of each spore. This was done, assuming the spore to be a prolate spheroid in shape (that is, a body derived by the elongation of a sphere in one diameter, according to the formula $\pi/6\ LB^2$, where L is the length and B is the breadth of the spore). Figures were calculated to the nearest $0{\cdot}001\mu$ and $0{\cdot}01\mu^3$.

For the purpose of representing size distributions graphically volumes were grouped in classes to the nearest cubic micron. For example, spores with volumes between $2{\cdot}500$ and $3{\cdot}500\mu^3$ have been given the volume of $3{\cdot}00\mu^3$ and so on. For each distribution the mean volume has been calculated, and in each case the standard error was modified by the use of Sheppard's correction in order to eliminate error introduced by grouping.

III. LIFE HISTORY

At 25° C. the life cycle is completed in 5 or 6 days. The general outline of the life cycle resembles that of *Nosema bombycis* and other species. In early stages of the cycle uni-nucleate and bi-nucleate forms measuring $1{\cdot}5$–$3{\cdot}0\mu$ predominate. (No 'planont' stage has been seen.) In later stages they are larger, mostly measuring $2{\cdot}5$–$3{\cdot}5\mu$. Schizonts with up to six nuclei have been seen, either rounded (4–$6{\cdot}5\mu$) or elongate (5–10μ). Mature meronts give rise to regularly oval sporonts (3–5μ). Each sporont gives rise to one spore.

The primary site of infection is in the longitudinal muscles, but in heavy infections most tissues may be invaded. The parasite can be transmitted through the eggs to the next generation.

IV. THE SPORE

(*a*) *Structure*

Fresh spores of this species are highly refractive and mostly ovoid in shape though a few larger spindle-shaped spores have been seen occasionally, but these are less refractive and are assumed to be abnormal. There is no indication that

the spore case is bivalved, even at magnifications of × 15,000–30,000, and its surface is smooth. Very little of the internal structure of the spore is visible without staining. One or two lighter areas, which are the vacuoles, appear at one or both ends of the spore, and the denser central part is the sporoplasm. The appearance of stained spores varies with the staining method: for example, with methylene blue, Giemsa, or Heidenhain's haematoxylin, as many as five or six densely staining bodies appear in the sporoplasm, whereas with carbol fuchsin or with Feulgen there are only two, arranged along the longer axis of the spore. As the two latter techniques are more specific for nuclear material than the others, the true number of nuclei is concluded to be two. No other details of spore structure can be made out with certainty. It is probable that the sporoplasm is girdle-shaped as in some other species so that the vacuoles are connected. The existence of a polar capsule is doubtful.

(*b*) *Hatching*

The polar filaments were extruded by means of ether, glacial acetic acid, saline, and the digestive juices of the hosts, but the most successful reagent was 3% 40-volume hydrogen peroxide, as used by Kudo (1918). Extrusion occurred more readily in each instance if the spores had been dried for several hours. On two occasions, using hydrogen peroxide, with air bubbles under the cover-slip, the process of filament extrusion was observed to occur in exactly the manner described by Gibbs (1953) for *Gurleya* sp., viz., as the filament grew longer it appeared as though a line were being drawn with a pencil, and when it was presumably fully extended a 'blob' appeared to issue from its distal end. Several other filaments were observed with similar terminal masses which measured about 2μ in diameter. On material stained with Giemsa after hydrogen peroxide treatment there were seen a number of small, irregularly shaped bodies, each containing two pink nuclei surrounded by blue cytoplasm, and measuring about 2μ: these were probably amoebulae, some of which retained the girdle-shape of the sporoplasm, showing a corresponding vacuole. By means of the electron microscope several spores were observed with extruded filaments and amoebulae attached, and in each case the spore was clearly empty. According to these observations it seems that eversion of the filament takes place, and that the sporoplasm leaves the spore through the filament and is injected into the gut epithelium of the host. Gibbs (1953), Korke (1916) and Oshima (1937), who have witnessed extrusion, also hold this view. Bailey (1955) provides further evidence for *Nosema apis* which also supports this theory of injection. The older view that the filament is merely an anchoring structure and that the sporoplasm leaves the spore after detachment of the filament and becomes a free-living 'planont' in the gut lumen is thus losing ground.

(*c*) *Size variation*

The first set of measurements was of spores taken from heavily infected longitudinal intersegmental muscles of a pupa. Some were measured fresh in insect Ringer solution (Ephrussi & Beadle, 1936), some fresh in distilled water, and others after being fixed in Bouin's picro-formol, stained in sections of tissue and mounted

in Canada balsam. Others were smeared, desiccated, and stored dry for 2 weeks, then measured in Ringer or in distilled water. Lastly, spores were stored in Ringer solution at 5° C. for 4 months and then measured. The results are plotted in Fig. 1 *A–F*. A constant feature of the results of these measurements is that the range in size of spores is considerable, regardless of medium. For instance, the minimum variation in length is 3·0μ, in breadth 0·9μ and in volume 8·0μ^3 (maximum 4·5μ,

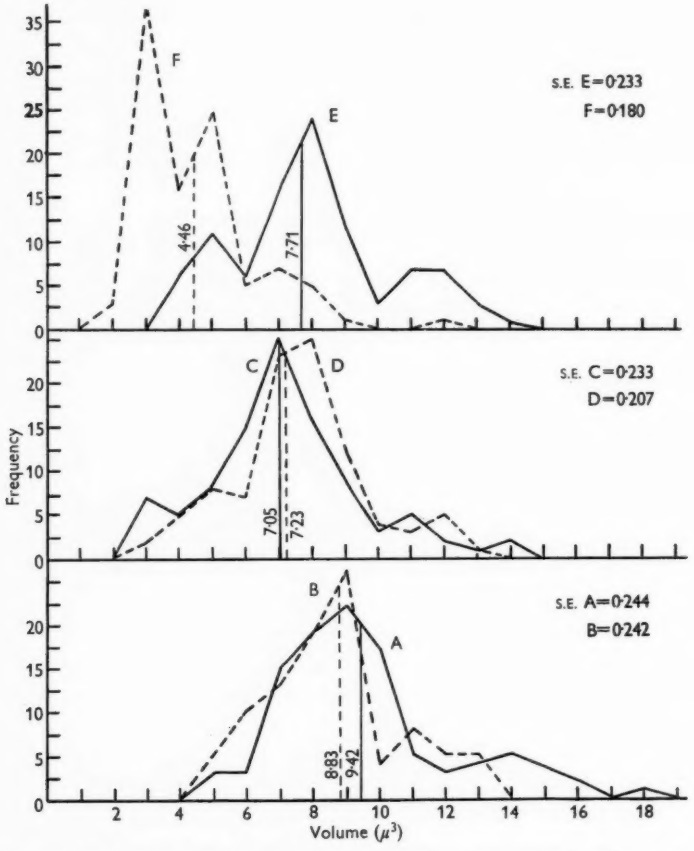

Fig. 1. A–F: graphs showing the size ranges of spores when measured in different conditions. A, spores fresh, measured in distilled water; B, spores fresh, measured in Ringer; C, spores desiccated, measured in Ringer; D, spores desiccated, measured in distilled water; E, spores stored in Ringer at 5° C. for 4 months, measured in Ringer; E, spores fixed, measured in Canada balsam.

1·1μ and 13μ^3 respectively). Besides this natural variation, the following further differences, caused by differences in treatment of the spores *in vitro*, can be seen only by comparison of the graphs.

Spores measured in distilled water are larger than those measured in Ringer. (Compare A with B, C with D.) Previous desiccation results in a reduction in

volume even when the spores are later placed in water or Ringer. (Compare A and B with C and D.) Fixed and stained spores (F) have a mean volume which is much smaller than any other, and approximately half the value for fresh spores (A and B). Storage of spores in Ringer solution at 5° C. for 4 months (E) causes a reduction in volume, indicating that this medium is slightly hypertonic.

Differences were found, though sometimes very slight, in spores from different situations in the host's body. Spores used in these measurements were taken from

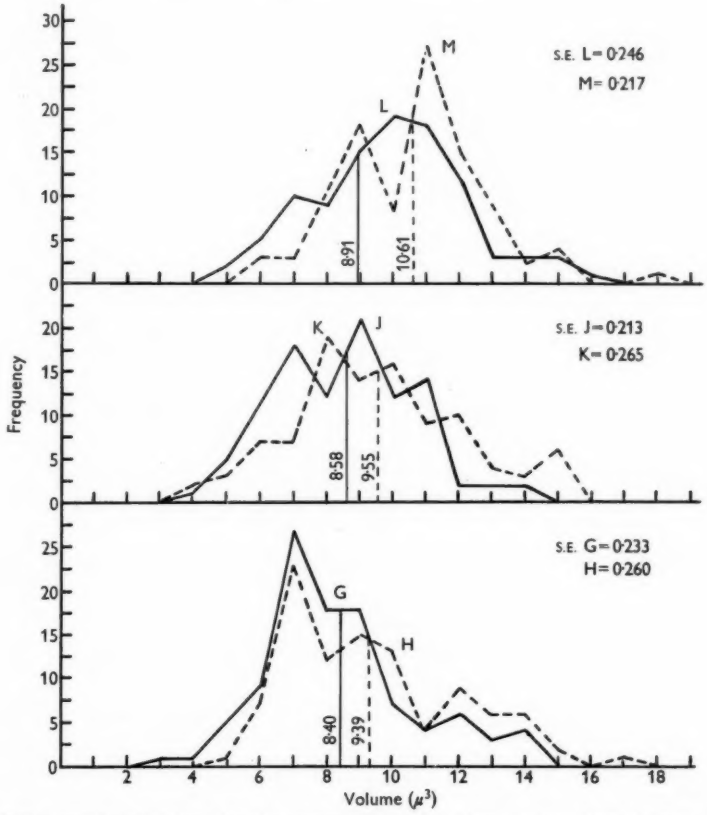

Fig. 1. G–M: graphs showing the size ranges of spores from various host tissues. G, from the gut wall of a mature larva; H, from a nerve ganglion of a mature larva; J, from abdominal muscle of a mature larva; K, from silk gland of a mature larva; L, from abdominal muscle of a young larva; M, from the gut wall of a young larva.

a fifth-instar larva and a third-instar larva which had been preserved in 5% formalin, and were measured in distilled water. The results are shown in Fig. 1, G–M. In the older larva, differences in mean volume were insignificant between the spores from gut epithelium and abdominal muscle, and between those from suboesophageal ganglion and silk gland, since the standard error was about $0{\cdot}2\mu^3$ in each case (Fig. 1, G, J, H, and K). Spores from nervous tissue and silk gland

were larger than those from gut wall and muscle. In the younger larva, spores from the gut wall were significantly larger than those from the muscles (Fig. 1, M, L). The younger larva produced larger spores than the older larva in corresponding tissues, and the over-all mean volumes were 9·76 and 8·96μ^3 respectively. This agrees with the findings of Fantham & Porter (1914) for spores of *N. bombi*, who compared dimensions, not volumes.

Comparison has so far been made chiefly on the basis of mean volume, but for comparison with other publications the range of value for length and breadth of the spores is shown in the following table:

Spores	Length (μ)	Breadth (μ)
Fixed and stained	2·7–5·7	1·1–2·0
Fresh in Ringer	3·2–7·7	1·4–2·3
Fresh in distilled water	3·6–8·1	1·6–2·7
From older larva (total)	3·2–7·2	1·4–2·5
From young larva (total)	3·4–6·8	1·4–2·5

Any one of these ranges embraces those given for a number of other species of *Nosema*.

V. DISCUSSION

It is concluded that the dimensions of the spores are affected by the age of the host animal, the particular host tissue in which the spores develop, the treatment given to spores after their removal from the host, and the medium in which they are measured.

The importance of these variations in the identification of species is at once apparent. For the great majority of recorded species the data given concerning the size of spores are insufficient, for the following reasons: the medium in which spores have been measured, or their condition, e.g. whether they have been kept for some time in preservative, has not generally been mentioned; in most cases only one measurement has been recorded, sometimes only the spore length, or, in species where a few abnormally large spores have been observed, these have been called 'macrospores' and two sizes recorded. Conet (1931), for example, gave two sizes for *Nosema herpobdellae*, $6 \times 3\mu$ and $10 \times 3{\cdot}5$–4μ—a difference of 4μ in length and $0{\cdot}5$–$1{\cdot}0\mu$ in breadth which lies within the ranges found in the present species. The same applies to *N. bryozoides* (Schröder, 1914) in which spores are recorded as $7{\cdot}8 \times 5{\cdot}6\mu$ and $10 \times 6\mu$.

Accurate identification of the present species of *Nosema* has not been possible because the range in the size of its spores overlaps or includes that given for a number of recorded species, and also because of deficiencies in many species descriptions. It shows most similarities to *N. bombycis*, which is cosmopolitan in occurrence: the life cycles are similar; both species infect all tissues of lepidopteran hosts, and neither of them is host-specific. (The present species has been maintained in a number of Lepidoptera and in the blowfly *Calliphora erythrocephala* (Finlayson & Walters, 1957).) Comparison on the basis of spore size is practically impossible, since five authors have independently given different measurements for *Nosema bombycis* (Balbiani, 1884; Thélohan, 1894; Stempell, 1909; Omori,

1912; Kudo, 1918), but one difference stands out, namely, that the spores of the present species are longer and narrower than those of *N. bombycis*.

The question of spore size does not affect the classification of the order Microsporidia as far as families and genera are concerned, for other characters are used, i.e. shape of the spores and spore development respectively. The facts which have come to light in the present study have, however, emphasized the need for a better system of species classification.

VI. SUMMARY

1. A brief outline of the life cycle of a species of *Nosema* parasitic in Saturniid moths is given. It is of the normal type.

2. The structure of the spore is described, and from electron microscopy evidence is presented which supports the theory that the sporoplasm is injected into the host's tissue.

3. The volume of the spore is affected by the ambient medium, by the pretreatments of fixation and of desiccation, by the site of development within the host and by the age of the host.

4. These results have been discussed with reference to the use of spore size as a diagnostic character in the classification of the Microsporidia. It is concluded that spore size is not a reliable character for taxonomic purposes.

The work was carried out while the author was in receipt of a Birmingham University Research Scholarship.

REFERENCES

Bailey, L. (1955). The infection of the ventriculus of the adult honeybee by *Nosema apis* (Zander). *Parasitology*, **45**, 86–94.

Balbiani, G. (1884). *Leçons sur les Sporozoaires*. Paris.

Blunck, H. (1954). Mikrosporidien bei *Pieris brassicae* L., ihren Parasiten und Hyperparasiten. *Z. angew. Ent.* **36**, 316–33.

Conet, M. A. (1931). *Nosema herpobdellae*, n.sp. microsporidie des Hirudinées. *Ann. Soc. Sci. méd. nat. Brux.* **51** B, 170–1.

Ephrussi, B. & Beadle, G. W. (1936). A technique of transplantation for *Drosophila*. *Amer. Nat.* **70**, 218.

Fantham, H. B. & Porter, A. (1914). The morphology, biology, and economic importance of *Nosema bombi* n.sp. parasitic in various bumble bees (*Bombus* spp.). *Ann. Trop. Med. Parasit.* **8**, 623–38.

Finlayson, L. H. & Walters, V. A. (1957). Abnormal metamorphosis in saturniid moths infected by a microsporidian. *Nature, Lond.*, **180**, 713–14.

Gibbs, A. J. (1953). *Gurleya* sp. (Microsporidia) found in the gut tissue of *Trachea secalis* (Lepidoptera). *Parasitology*, **43**, 143–7.

Korke, V. T. (1916). On a *Nosema* (*Nosema pulicis* n.sp.) parasitic in the dog flea (*Ctenocephalus felis*). *Indian J. Med. Res.* **3**, 725–30.

Kudo, R. (1918). Experiments on the extrusion of polar filaments of Cnidosporidian spores. *J. Parasit.* **4**, 141–7.

Kudo, R. (1924). A study of the Microsporidia. *Illinois Biol. Monogr.* **9**, 79–268.

Omori, J. (1912). Zur Kenntnis der Pebrine-Erreger, *Nosema bombycis* Nägeli. *Arb. Gesundh-Amt., Berl.*, **40**, 108–22.

Oshima, K. (1937). On the function of the polar filament of *Nosema bombycis*. *Parasitology*, **29**, 220–4.

PEREZ, C. (1905). Microsporidies parasites des crabes d'Arcachon. Note préliminaire. *Bull. Sta. biol. Arcachon*, **8**, 15–36.

RYCKEGHEM, J. VAN (1930). Les Cnidosporidies et autres parasites du *Gammarus pulex*. *Cellule*, **39**, 399–419.

SHORTT, H. E. & COOPER, W. (1948). Staining of microscopical sections containing protozoal parasites by modification of McNamara's method. *Trans. R. Soc. Trop. Med. Hyg.* **41**, 427.

SHRÖDER, O. (1914). Beiträge zur Kenntnis einiger Microsporidien. *Zool. Anz.* **43**, 320–7.

SHUTE, P. G. (1955). An improved technique for staining blood films with Giemsa stain. *Nature, Lond.*, **175**, 727.

STEMPELL, W. (1909). Über *Nosema bombycis* Nägeli. *Arch. Protistenk.* **16**, 281–358.

STEMPELL, W. (1919). Untersuchungen über *Leptotheca coris* n.sp. und das in dieser schmarotzende *Nosema marionis* Thélohan. *Arch. Protistenk.* **40**, 113–57.

THÉLOHAN, P. (1894). Sur la présence d'une capsule à filament dans les spores des microsporidies. *C.R. Acad. Sci., Paris*, **118**, 1425–7.

WILLIAMS, C. M. (1952). Physiology of insect diapause. IV. The brain and prothoracic gland as an endocrine system in the Cecropia silkworm. *Biol. Bull., Woods Hole*, **103**, 120–38.

(*MS. received for publication* 12. IV. 1957.—Ed.)

A NOTE ON A SPECIES OF *SYNGAMUS* FOUND IN THE INTESTINE OF *TURDUS PILARIS*

By D. L. LEE

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(With 5 Figures in the Text)

A male and a female of a species of *Syngamus* were found *in copula* in the small intestine, about 1 in. from the gizzard, of a fieldfare (*Turdus pilaris*) which had been shot accidentally. The nematodes were well preserved and were undamaged. Eighty-four small tapeworms, of two different genera, were also present in the intestine. Neither the tapeworms nor the nematodes were attached to the intestinal wall.

The bird had lived for about 2 min. after being shot and during this period choking and gaping movements of the beak were noticed. About 5 hr. after death the bird was put into 5–10 % formalin after the abdomen had been slit open; and it was later given to me for examination for parasites.

The nematodes were found in an unusual position for species of *Syngamus* and, as they show some morphological differences from the established species, their main characteristics will be described before discussing their systematic position.

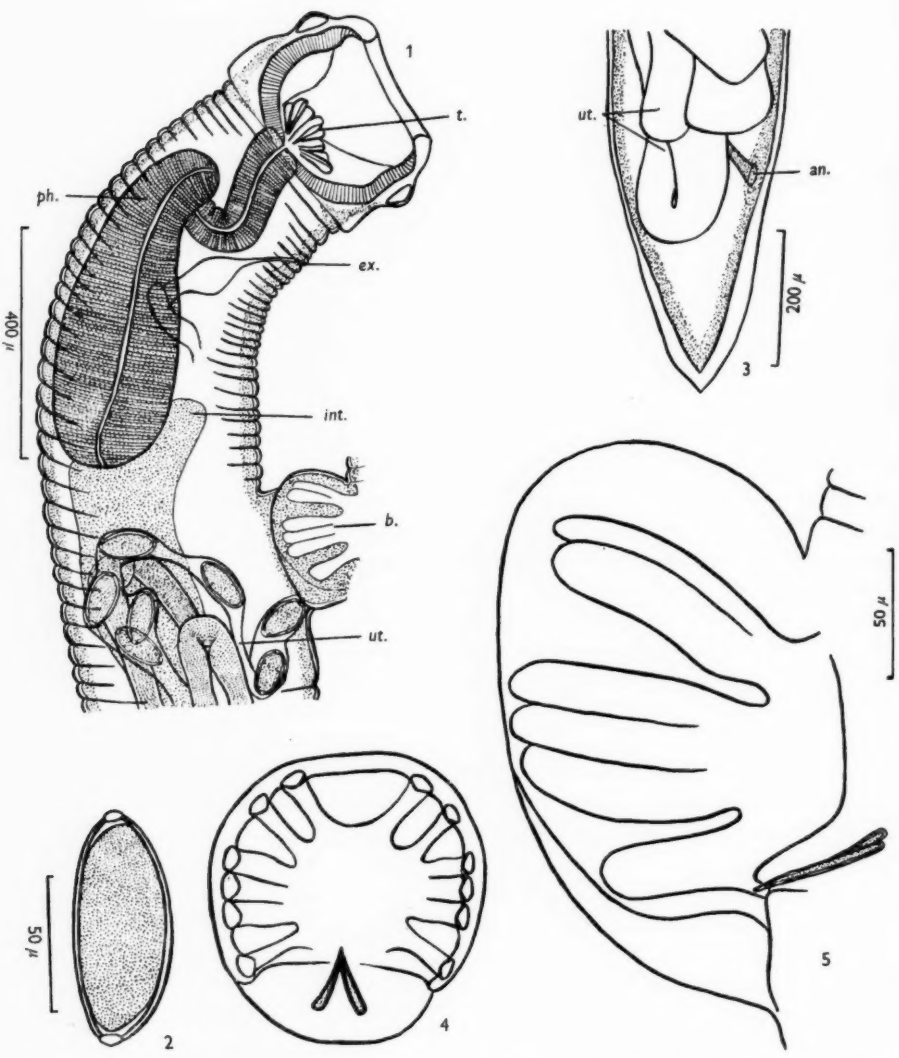
Description of male (Figs. 4, 5): length 1·4 mm., greatest width 216μ. Buccal capsule 125μ in depth, 100μ in greatest inside diameter, 187μ in external diameter. Oesophagus 600μ long, 130μ in maximum diameter. Nerve ring 420μ from anterior end. Excretory pore was not located. Caudal bursa, 230μ in diameter; dorsal ray deeply bifurcated with each bifurcation simple; externo-dorsal ray simple and equal in length to dorsal ray; lateral rays mutually contiguous and parallel; ventral ray slightly shorter than other rays. Spicules, two, equal, 53μ long (Fig. 5).

Description of female (Figs. 1–3): length 6 mm., greatest width 375μ. Buccal capsule 190μ in depth, 212μ in greatest inside diameter, 287μ in external diameter. Eight buccal teeth are present. Oesophagus 750μ long, 187μ in maximum diameter. Nerve ring was not located. Excretory pore 280μ from anterior end. Vulva 900μ from anterior end. Uterine coils end 217μ from posterior end. Anus 327μ from posterior end. Eggs 87–92 × 40–42μ. They are unsegmented in the uterus, have two opercula and are slightly asymmetrical (Fig. 2).

The bursa is very similar to that of *Syngamus gracilis* Chapin, 1925, the spicules, however, are the correct shape and size for *S. parvus* Chapin, 1925, being short and equal, whilst those of *S. gracilis* are longer, unequal and with the right spicule bent. As the bursa of all species of *Syngamus* is a very variable structure (Lewis, 1928), identification of the specimens as *S. parvus* is based on the shape and size of the spicules, which distinguish them from *S. gracilis*, and on the unsegmented ova in the uterus which distinguish them from *S. trachea* (Montagu, 1811) Chapin, 1925.

Turdus pilaris is a new host for this species, those described by Chapin (1925) were from the trachea of *Nucifraga caryocatactes*.

The fieldfare had been shot through the lung and it is possible that the nematodes were coughed up from the trachea when the bird was choking, swallowed, and then passed into the intestine. It is difficult, however, to see how they would escape



Figs. 1–5. *Syngamus parvus*. Fig. 1. Anterior end of female, lateral view. Fig. 2. Egg, lateral view to show asymmetry. Fig. 3. Posterior end of female, lateral view. Fig. 4. Ventral view of caudal bursa of male. Fig. 5. Lateral view of bursa and spicules of male. Abbreviations: *an.* anus; *b.* bursa of male; *ex.* excretory pore; *int.* intestine; *ph.* pharynx; *t.* buccal teeth; *ut.* uterus.

injury in the gizzard. The other alternative is that the nematodes were actually living in the intestine, but this is even more unlikely as all other species of *Syngamus* are found living in the respiratory tract or in the middle ear (Faust & Tang, 1934) of their host. The only other case of a species of *Syngamus* being found in the intestine is the *Distomum lineare* described as a trematode by Rudolphi (1793, quoted by Ortlepp, 1923). His description of the parasite corresponds to that of *S. trachea* with which it is considered to be synonymous. Ortlepp (1923) takes the view that Rudolphi was dealing with a displaced *S. trachea*, but he also found it hard to believe that the nematodes would not be injured during their passage through the gizzard.

Although in the present case the specimens identified as *S. parvus* were found in the intestine of a fieldfare this is only the second record of a species of *Syngamus* from the intestine of a bird, and considering also the manner in which the bird died, I conclude that the specimens were not in their normal habitat but had been displaced from the respiratory tract.

I wish to thank Mr D. W. Sutcliffe who supplied the material, and Dr P. A. Clapham for her advice on the identification of the specimens. The work was done during the tenure of a Research Studentship awarded by the Department of Scientific and Industrial Research.

REFERENCES

Chapin, E. A. (1925). Review of the Nematode Genera *Syngamus* Sieb. and *Cyathostoma* Blanchard. *J. Agric. Res.* **30**, 557–70.

Faust, E. C. & Tang, C. (1934). A new species of *Syngamus* (*S. auris*) from the middle ear of the cat in Foochow, China. *Parasitology*, **26**, 455–9.

Lewis, E. A. (1928). Observations on the morphology of *Syngamus* of some wild and domestic birds. *J. Helminth.* **6**, 99–112.

Ortlepp, R. J. (1923). The life history of *Syngamus trachealis* (Montagu), v. Siebold, the gape-worm of chickens. *J. Helminth.* **1**, 119–40.

Rudolphi, C. A. (1793). *Observationes circa Vermes Intestinales.* Gryphiswaldiae, p. 29. (Not seen in original, see Ortlepp, 1923.)

(*MS. received for publication* 27. iv. 1957.—Ed.)

A REVIEW OF SOME TICKS (ACARINA: IXODIDAE) OF SEA BIRDS, INCLUDING A CONSIDERATION OF OTHER COGNATE MATERIAL. I

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(With 13 Figures in the Text)

The *Ixodes* ticks of sea birds are a very interesting ecological and morphological group which have a wide distribution in both the southern and northern hemispheres. The genus *Ixodes* on sea birds was reviewed by Zumpt (1952) who listed the following species: *Ixodes uriae* (=*Ceratixodes uriae*) White, 1852; *I. kerguelenensis* André & Colas-Belcour, 1942; *I. auritulus* Neumann, 1904; *I. rothschildi* Nuttall & Warburton, 1911; *I. murreleti* Cooley & Kohls, 1945; *I. unicavatus* Neumann, 1908; *I. signatus* Birula, 1895 and *I. neumanni* Nuttall & Warburton, 1911. Further investigation of material from all parts of the world has led me to believe that a redescription of the type specimens and a review of as many specimens as possible of various species is desirable to clear up existing inaccuracies. In this I have been materially assisted by Dr F. Zumpt, Medical Research Council, South Africa; Dr L. J. Dumbleton, Wellington, New Zealand; Dr G. Owen Evans, Department of Zoology, British Museum (Natural History); Mr G. B. Thompson, Cambridge; Mr Glen M. Kohls, Rocky Mountain Laboratory, Montana, U.S.A.; Dr Gertrud Theiler, Onderstepoort Research Station, South Africa; Professor Brizard, École Nationale Vétérinaire de Toulouse, France, and Dr Marc André, Muséum d'Histoire naturelle, Paris.

Ixodes rothschildi Nuttall & Warburton 1911

The female and nymph of this species were previously redescribed by Arthur (1953), larva described by Arthur (1955*a*).

Male

Source of material. Five males and thirteen females in burrows of *Fratercula artica grabae*, Skokholm, Wales, 15 June 1955 (G. B. Thompson collection, 1955/113).

Description. Length, tips of scapulae to posterior margin of body (Fig. 1), 2·76 mm.; width 2·0 mm. Suboval, widest about midlength. Colour, scutum and ventral plates reddish brown, palps and legs yellowish brown, basis capituli darker than palps and legs.

Capitulum. Length, tips of palpi to posterior margin of basis 0·51 mm.; greatest width of basis behind palpal insertion 0·36 mm. Basis narrowed behind, posterior margin concave, strongly and sharply elevated; cornua absent (Fig. 4). Palpi short, broad, many hairs of variable length; segments 2 and 3 not separated by a distinct suture line, their combined length about 0·295 mm In ventral view, the basis long

(about 0·32 mm.), broadest (about 0·36 mm.) behind palpal insertion, convex transverse groove, postero-laterally more heavily sclerotized and slightly elevated 'spine'-bearing areas (Fig. 3). Palpal segment 1 broader than long and ridged ventrally.

Hypostome (Fig. 5). Broad, shape as figured. Apically indented, distally without lateral teeth, proximally with short lateral teeth, median denticles as small crenulations, passing back obliquely proximally leaving a median triangular unarmed area. Length of toothed portion 0·16 mm.

Scutum (Fig. 1). Surface essentially glabrous, but a few very short hairs evident under high magnification, smooth, shining. Surface strongly arched along the midline. Cervical grooves very short, deep. Lateral carinae absent. Pseudoscutum not evident. Punctations few, very small and shallow, widely separated. Lateral border wide, punctate, with few short hairs.

Ventral plates (Fig. 2). Median plate about one and a quarter times as long as broad, and about twice as long as anal plate, punctations very few, very small and widely dispersed. Under high magnification few, very short hairs posteriorly. Anal plate broadly rounded in front, sides subparallel for most of its length, slightly indented posteriorly, but not reaching to the hind margin of the body; hairs few and longer than on median plate. Adanal plates wider in front and with hairs similar to those on the anal plates; punctations of two kinds, an anterior narrow transverse band of larger deeper punctations, otherwise similar to those on median plate. In all five specimens, even after drying, the limits of these ventral plates are irregular and variable, but in all the posterior edge of the median plate never extends to the anterior edge of the anal plate.

Legs. Of moderate length and breadth. Coxae (Fig. 7) with long spinose hairs, arranged distally and in a row of three or four along the posterior edge; surface undulate particularly on coxa I, distal margins strongly convex producing postero-lateral tuberosities. Coxa I almost triangular, lacking spurs, coxae II and III with slight postero-internal marginal saliences. Trochantal spurs absent. Tarsi tapering from proximal to distal end, all tarsi abruptly narrowed distally. Haller's organ forming a concavity. Length of tarsus I (Fig. 6*a*), 0·6 mm., metatarsus I, 0·27 mm. Length of tarsus IV (Fig. 6*b*), 0·65 mm., metatarsus IV, 0·5 mm.

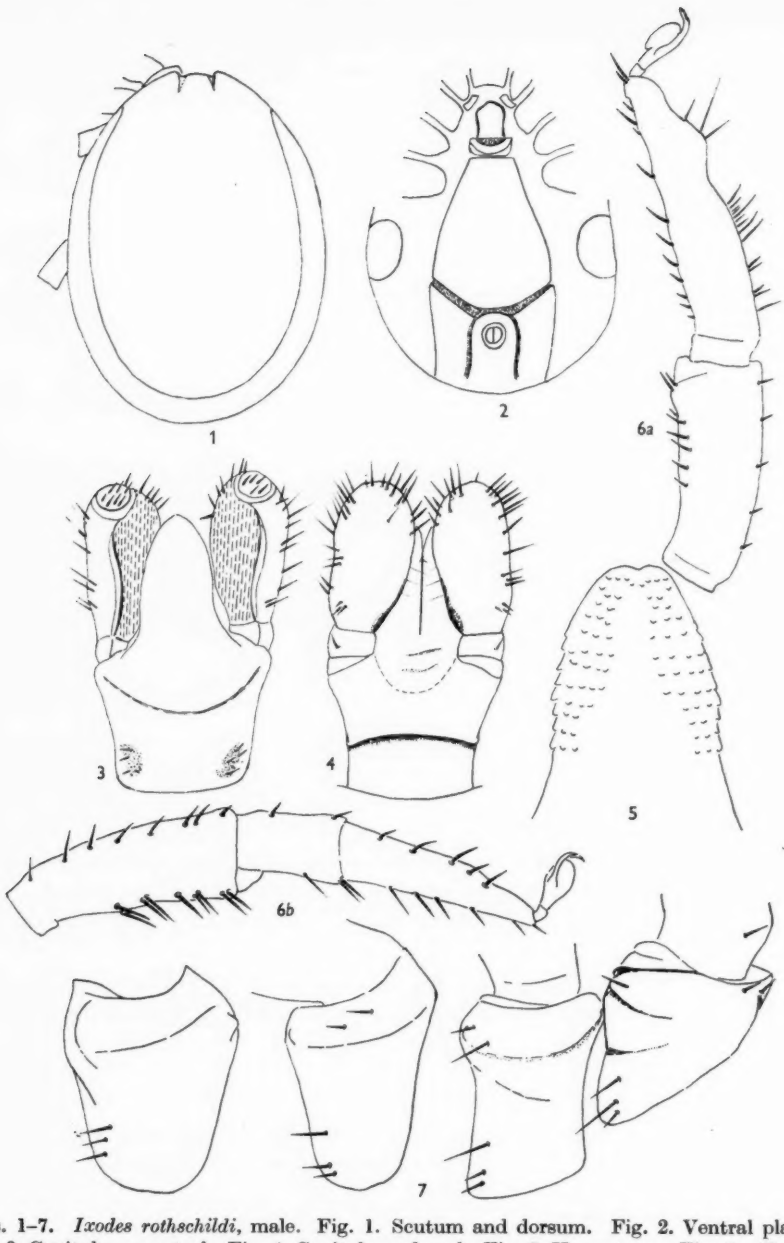
Spiracular plate (Fig. 2). Shape as figured. Greatest dimension 0·39 mm. Long axis corresponds with long axis of body, macula anteriorly placed.

Related species

Females of *I. rothschildi* are readily distinguished from *I. auritulus* and *I. percavatus* by the absence of an anterior mesial projection on palpal article I, and from *I. kohlsi* Arthur in the arrangement of teeth on the hypostome; the shape of the anal grooves; the tarsi which taper more abruptly than do those of *I. rothschildi*, and Haller's organ which is a closed capsule in *I. kohlsi* (Arthur, 1955*b*).

Males of *I. rothschildi*, *I. auritulus** and *I. kerguelenensis* (of André & Colas Belcour, 1942) all have a similar general facies, *I. auritulus* is the smallest and

* This refers to the male collected by Dumbleton in Snares Island, New Zealand, and ascribed to *I. auritulus* (see Dumbleton, 1953, p. 9.)



Figs. 1–7. *Ixodes rothschildi*, male. Fig. 1. Scutum and dorsum. Fig. 2. Ventral plates. Fig. 3. Capitulum, ventral. Fig. 4. Capitulum, dorsal. Fig. 5. Hypostome. Fig. 6*a*. Tarsus and metatarsus I. Fig. 6*b*. Tarsus and metatarsus IV. Fig. 7. Coxae.

I. kerguelenensis (of André & Colas Belcour) the largest. This latter species is readily separated from the other two males in having Haller's organ superficially located on tarsus I, but in both *I. auritulus* and *I. rothschildi* this organ is of the concavity type (see Arthur, 1956). In *I. auritulus* and *I. kerguelenensis* the anal plate is long and narrow, in *I. rothschildi* this plate is short and broad; the hypostome of *I. auritulus* differs markedly from either of the others in the arrangement and form of the denticles, while the shapes and dentition of the hypostomes of *I. rothschildi* and *I. kerguelenensis* permit ready recognition (see key to males).

Remarks

This species was originally described as a variety of *I. percavatus* by Nuttall & Warburton (in Nuttall, Warburton, Cooper & Robinson, 1911). Zumpt (1952) and Arthur (1953, but submitted for publication in 1952), working independently, arrived at the conclusion that the variety *rothschildi* was sufficiently distinct from *percavatus* to merit specific rank. On further revision of the status of *rothschildi*, I am now of the opinion that the ticks referred by Zumpt to *I. rothschildi* differ from those ticks I called *rothschildi*. The descriptions of the female, nymph and larva of *I. rothschildi* given elsewhere (Arthur, 1953) were based on the two females and three nymphs (N. 634) which were the type material for Nuttall & Warburton's variety. Having examined the female off *Diomedea chlororhynchus* which Zumpt assigned to *rothschildi* I cannot agree that it is referable to the variety of that name as given by Nuttall & Warburton (1911). Zumpt's specimen is, in my opinion, a new species to which I give the name *Ixodes diomedeae* (see p. 128).

Hosts

Puffin (species not stated); *Fratercula arctica grabae* (Puffin); *Puffinus p. puffinus* (Manx shearwater); *Phalacrocorax a. aristotelis* (Shag nestling).

Distribution

Apart from the type species for which no locality data are available, the existing records are all from islands off the British Coast. Doubtless further investigation will extend the geographical range of this species.

Biology

Very little is known about the biology of this species. Females, nymphs and larvae have been collected from the head, around the eyes and less frequently on the wing membrane of the hosts. The activity of these parasitic stages in any numbers on hosts have so far been observed between April and July. Thirteen gorged females were collected in June from the burrows of puffins, suggesting a recent drop-off from the hosts. The details of these collections are given by Arthur & Thompson (1953), Thompson & Arthur (1955, 1956). Until the presentation of the data in this paper males were unknown. Five males were collected from the burrows of *Fratercula arctica grabae*, and their apparent non-occurrence on birds together with the poorly developed hypostome suggest very strongly that the males live in the burrows or the nests of their hosts. It seems likely that copulation takes place off the host.

KEY TO MALES

(Only males of *I. rothschildi*, *I. auritulus* and *I. kerguelenensis* are at present known.)

1. Hypostome with crenulations extending along the whole length. 2
— Hypostome with crenulations distally, strongly toothed basally. **I. auritulus**
— Dorsal ridge subangular postero-laterally; palps tapering quite strongly to base; anal groove reaching posterior margin; hypostome subparallel. **I. kerguelenensis**
— Dorsal ridge slightly concave; palps broad for most of their length and not tapering strongly to their insertion; anal groove not reaching posterior margin; hypostome broad basally narrowing strongly to the apex. **I. rothschildi**

Ixodes diomedeae n.sp.

Holotype. One female, from *Diomedea chlororhynchus*, Nightingale Island, Tristan da Cunha, April 1950. Deposited in the Institute of Medical Research, Johannesburg, South Africa.

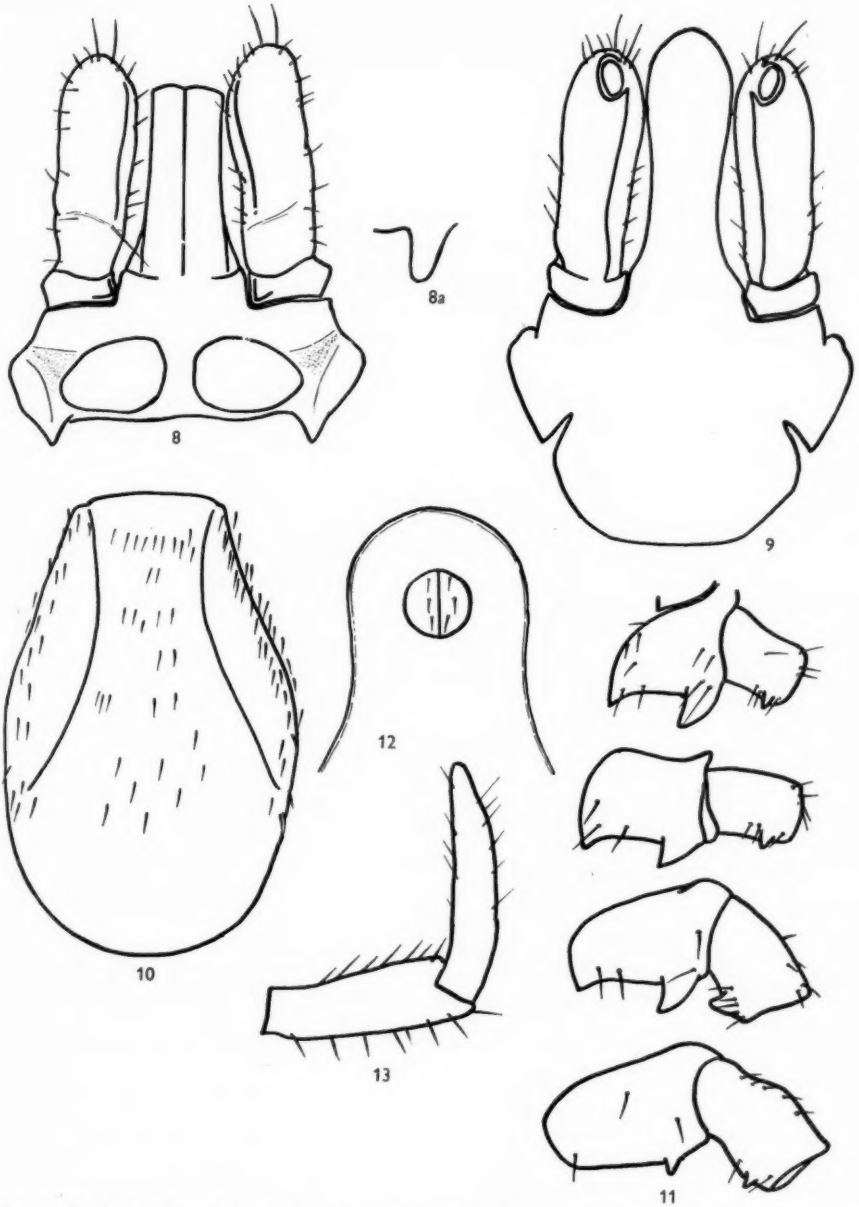
Description of female. Partly gorged female, length 8 mm., greatest breadth 5 mm., elongate oval in outline. Colour of sclerotized parts medium to dark brown.

Capitulum. Length, tips of palpi to posterior margin of basis, 0·73 mm., greatest width 0·72 mm. Surface flat except for a depression lateral to the porose areas (stippled in Fig. 8). Posterior margin undulate, cornua long, rounded apically and almost vertical to the plane of the rest of the basis (Fig. 8*a*). The sides strongly divergent and strongly angled at about the midlength of the basis, thereafter converging by a rectilinear margin to the palpal insertion. Porose areas large, moderately shallow, transversely ovate (almost pyriform). Palpi short, broad with both inner and outer edges almost straight and parallel; articles II and III not separated by a suture line; combined length of these articles 0·45 mm., greatest breadth 0·16 mm. Hairs variable in length, the longer ones dorsally and proximally, and at the apex. Palpal article I broader than long, lateral margin strongly angled and bearing a broad spur meso-dorsally. In ventral view basis broadest in front of the auriculae, latter short, pointed; constriction moderate (Fig. 9). Posterior margin straight, postero-lateral margins angular.

Hypostome. Longer than palpi, 0·5 mm.; in this specimen it is damaged, but as far as can be ascertained the apex is rounded, and behind the corona the dentition is arranged in 2 rows of 4/4 files, 3 rows of 3/3 files and 5 rows of 2/2; distal teeth sharp, proximal teeth lobate.

Scutum. Length 1·75 mm., breadth 1·1 mm., posterior half broadly rounded, narrowing quite appreciably in front as shown in Fig. 10. Surface convex antero-posteriorly. Cervical grooves as broad shallow valleys extending well back, but not attaining postero-lateral margins. Punctations of two kinds being small, shallow, rather sparse and lacking hairs, and large and shallow, bearing hairs; the large punctations are particularly abundant beyond the cervical grooves and less numerous in the median and posterior region. Scapulae and emargination lacking; lateral carinae absent.

Legs. Of moderate length and breadth. Coxa I with stout, curved external



Figs. 8–13. *Ixodes diomedeae* n.sp., female. Fig. 8. Capitulum, dorsal, cornua foreshortened. Fig. 8*a*. Cornua seen in horizontal view. Fig. 9. Capitulum, ventral. Fig. 10. Scutum. Fig. 11. Coxae and trochanters I–IV. Fig. 12. Anal groove. Fig. 13. Tarsus and metatarsus IV.

spur, internal spur short and sharp. External spurs on coxae II and III large, that on coxa IV small; internal spurs on coxae II short, sharp but nevertheless distinct, those on III reduced to marginal saliences and absent on coxae IV. Trochanter spurs pointed and present on all legs. Tarsi I missing, tarsus IV tapering gradually to the apex, length 0·9 mm. (Fig. 13).

Spiracular plate. Transversely oval, macula anteriorly placed; goblets small and numerous.

Anal groove. Broadly rounded in front, arms diverging behind but not reaching posterior margin.

Genital orifice. On a level with the posterior edges of coxae II.

Postscutal area. Bears hairs dorsally which are longer than those on the scutum; those on the venter shorter.

Related species

This species, although previously ascribed to *I. rothschildi* by Zumpt (1952), differs from the type and from other specimens of *I. rothschildi* in the shape of the basis capituli and palps, in the angled lateral margins of palpal article I, in the shape and orientation of the cornua and the auriculae. Differences are also evident in the scutal pattern and in the spiracular plate, though the latter may well fall within the intraspecific range of variability.

SUMMARY

1. The male of *Ixodes rothschildi* is described for the first time from the burrows of *Fratercula arctica grabae* at Skokholm, Wales.

2. The species described as *I. rothschildi* by Zumpt is now considered as a new species, *I. diomedeae*.

REFERENCES

ANDRÉ, M. & COLAS-BELCOUR, J. (1942). Sur une nouvelle variété d'*Ixodes canisuga* (Acarien). *Bull. Mus. Hist. nat., Paris*, **14**, 261–3.

ARTHUR, D. R. (1953). The systematic status of *Ixodes percavatus* var. *rothschildi* Nuttall & Warburton. *Parasitology*, **43**, 222–6.

ARTHUR, D. R. (1955*a*). The larva of *Ixodes rothschildi* Nuttall & Warburton, 1911. *Ann. Mag. Nat. Hist.* (Ser. 12), **8**, 711–12.

ARTHUR, D. R. (1955*b*). *Ixodes kohlsi*, a new species of tick from Australia. *J. Parasit.* **41**, 18–23.

ARTHUR, D. R. (1956). The morphology of the British Prostriata with particular reference to *Ixodes hexagonus* Leach, III. *Parasitology*, **46**, 261–307.

ARTHUR, D. R. & THOMPSON, G. B. (1953). Records of ticks collected from birds in the British Isles. *Ann. Mag. Nat. Hist.* (Ser. 12), **6**, 797–800.

DUMBLETON, L. J. (1953). The ticks (Ixodoidea) of the New Zealand sub-region. *Cape Exped. Ser. Bull.*, no. 14, 1–28, 1–4 plates.

NUTTALL, G. H. F., WARBURTON, C., COOPER, W. F. & ROBINSON, L. E. (1911). *Ticks. A monograph of the Ixodoidea*, part II. Cambridge University Press.

THOMPSON, G. B. & ARTHUR, D. R. (1955). Records of ticks collected from birds in the British Isles (2). *Ann. Mag. Nat. Hist.* (Ser. 12), **8**, 57–60.

THOMPSON, G. B. & ARTHUR, D. R. (1956). Records of ticks collected from birds in the British Isles (3). *Ann. Mag. Nat. Hist.* (Ser. 12), **9**, 385–90.

ZUMPT, F. (1952). The ticks of sea birds. *Aust. Nat. Antarc. Res. Exp.* (Ser. B), **1**, 12–20.

(*MS. received for publication* 8. v. 1957.—Ed.)

CYTOLOGICAL AND HISTOCHEMICAL CRITERIA FOR EVALUATING DEVELOPMENT OF TREMATODES AND PSEUDOPHYLLIDEAN CESTODES *IN VIVO* AND *IN VITRO*

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(With 2 Figures in the Text)

INTRODUCTION

Improvements in techniques for *in vitro* cultivation of parasitic helminths depend for their evaluation on criteria for the recognition of the degree of development taking place under a particular set of environmental conditions. Such criteria must be clearly related to the 'normal' pattern of development in a susceptible host, using the term 'normal' in an operational sense to denote a maturation which culminates in the production of eggs capable of completing the life cycle *in vivo*.

The most satisfactory criteria are those which are (*a*) precisely definable (e.g. the appearance of active spermatozoa), (*b*) readily recognizable, either by direct observation or by means of simple techniques, and (*c*) cover the whole range of the maturation processes. The last condition is especially important, and criteria for very early stages must be established if the prolongation of unsuccessful experiments is to be avoided.

Only in nematodes is it usually possible to use visual criteria for the phases of maturation on account of the relative transparency of the larval forms. In trematodes and cestodes, the opaque nature of the tissues makes direct observation of these phases impossible, and a detailed study of the tissues or organs at a cytological, histological or histochemical level becomes necessary when the choice of suitable criteria is being considered.

Since the early stages of maturation are characterized by the occurrence of intensive mitotic activity, it has been suggested that an estimate of this activity, as demonstrated by colchicine inhibition, could serve as a useful criterion for early helminth development (Smyth, 1956*a*), and Bell & Hopkins (1956) have attempted to apply this method to assess the development *in vitro* and *in vivo* of *Diplostomum phoxini*. The application of this technique is considered in further detail here.

For the later maturation stages, resulting in the production of gametes and the elaboration of the materials forming the egg-shell, and for the maintenance of the 'normal' adult condition, other criteria of a cytological or histochemical nature are indicated. The establishment and application of such criteria are dealt with in the present paper. It is known that the pattern of genitalia maturation in pseudo-

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† Grateful acknowledgement is made to the Royal Society and to the Medical Research Council of Ireland for generous grants-in-aid for apparatus and materials used in this work.

phyllidean cestodes and digenetic trematodes is basically the same (Smyth, 1956*b*) and it will be shown that closely similar criteria may be used for the maturation phases of these two groups. Most of the methods employed have been developed during attempts to cultivate to sexual maturity *in vitro* two organisms: (*a*) the plerocercoid of the pseudophyllidean cestode *Diphyllobothrium* sp., and (*b*) the metacercaria of the strigeid trematode *Diplostomum phoxini*; but some of the methods used may find wider application to *in vitro* helminth work in general.

The data given here are concerned mainly with the establishment of criteria; detailed results of *in vitro* experiments will be published separately.

II. MATERIAL AND METHODS

Plerocercoid larvae of *Diphyllobothrium* sp. were obtained from the viscera and body cavity of the stickleback. Adult worms were obtained by feeding plerocercoids to rats with a stomach tube according to the method of Archer & Hopkins (1957) who have given a detailed account of the growth in this host. We are indebted to Dr Hopkins for details of this technique. By performing autopsies at suitable times, worms in all stages of development, from plerocercoid to sexually mature strobila, could be obtained. For morphological studies from whole mounts, worms were fixed in Bouin's fluid and stained in Gower's carmine. A set of 'standards' showing the expected extent of *in vivo* development each day was therefore available. Sterile *in vitro* cultures of plerocercoids were obtained by the method previously described (Smyth, 1947). In view of the uncertainties regarding the taxonomy of the *Diphyllobothridae* (Archer & Hopkins, 1957; Rausch, 1956) the species has not been determined with certainty, but it is tentatively identified as *D. dendriticum*. Maturation in the strain of albino rats used required only 7 days, a figure close to that obtained by Archer & Hopkins (1957) and Kuhlow (1953).

Metacercariae of *Diplostomum phoxini* were obtained from the brain of minnows. Adult trematodes were obtained by feeding whole minnow heads to ducks and carrying out autopsies at suitable intervals. Whole mounts were prepared as for *Diphyllobothrium* or otherwise as indicated in the text. For *in vitro* work, the surface of the skin was sterilized with absolute alcohol and iodine, the entire brain removed, teased in Tyrode's solution, and the released metacercariae transferred to suitable containers for culture. This method for obtaining sterile larvae is basically that of Bell & Hopkins (1956), but the media used and the conditions of culture differed considerably from those used by these workers; details will be published elsewhere (Bell & Smyth, 1957).

III. *IN VIVO* DEVELOPMENT

The processes taking place in the maturation of a pseudophyllidean plerocercoid to an adult cestode or a metacercaria to an adult trematode are essentially concerned with the development of genitalia for the elaboration of the egg. In many pseudophyllidean cestodes and most trematodes the egg-shell is composed of sclerotin, a quinone-tanned protein, and available evidence (Stephenson,1947;

Romanini, 1947; Vialli, 1950; Smyth, 1954*b*, 1956*b*; Gonnert, 1955; Johri & Smyth, 1956) suggests that the method of formation in these two groups is basically the same, though differing in detail.

It is thus possible to give a generalized picture of maturation which is common to both *Diphyllobothrium* and *Diplostomum*. For convenience, development has been divided into a number of phases, the transition between each phase being marked by a change over to different criteria. It is recognized, as in all embryonic development, that we are dealing with a continual process in which the borderlines between some phases are not readily definable. The phases selected are as follows:

Phase 0. *Uncultured metacercaria or plerocercoid.* Larvae consist of a mass of undifferentiated cells which show no traces of genital primordia (Fig. 1A).

Phase 1. *Cell multiplication.* The provision of a large number of cells for organogeny. Characterized by intensive mitotic activity.

Phase 2. *Segmentation or body shaping.* The division of the cestode strobila into proglottides, or the trematode into an adult body outline.

Phase 3. *Organogeny.* Appearance of genital primordia and their transformation into organs recognizable by their shape or position. No recognizable cellular differentiation (Fig. 1B).

Phase 4. *Early gametogeny.* The occurrence of the early stages in spermatogenesis, oogenesis, and vitellogenesis.

Phase 5. *Late gametogeny.* Maturation of spermatozoa and ova (Fig. 1C).

Phase 6. *Egg-shell formation and vitellogenesis.* Appearance of egg-shell precursors and yolk (Fig. 1D).

Phase 7. *Oviposition.* The appearance of a fully formed shelled egg (Fig. 1E).

Phase 0. *Uncultured larvae*

In uncultured larvae of *Diphyllobothrium*, tissues are undifferentiated apart from the presence of bothria (Fig. 1A). In *Diplostomum*, only a minute gut, suckers and excretory vesicle are present (Fig. 1A). In sections, or aceto-orcein squashes, except for an occasional mitosis, nuclei show no activity. Larvae at this stage may be regarded as embryos whose later development has been retarded.

Phase 1. *Cell multiplication*

On ingestion by a warm-blooded host, further development is initiated by the sudden rise in temperature and characterized by an outburst of intense mitotic activity. The mitoses may be conveniently detected by treating larvae with colchicine, which causes inhibition at metaphase, and preparing aceto-orcein squashes (Smyth, 1956*a*). In *Diplostomum*, the small size of the organism permits every mitosis to be counted, but in *Diphyllobothrium*, and larger helminths in general, the size excludes this possibility and resource has to be made to methods of approximation.

In such cases the method recommended is to count the number of inhibited mitoses in an agreed number of oil immersion fields of the squash; thirty fields have been found generally satisfactory. The accurate measurement of mitotic activity in terms of units such as the 'mitotic index' or the 'cell doubling time' is

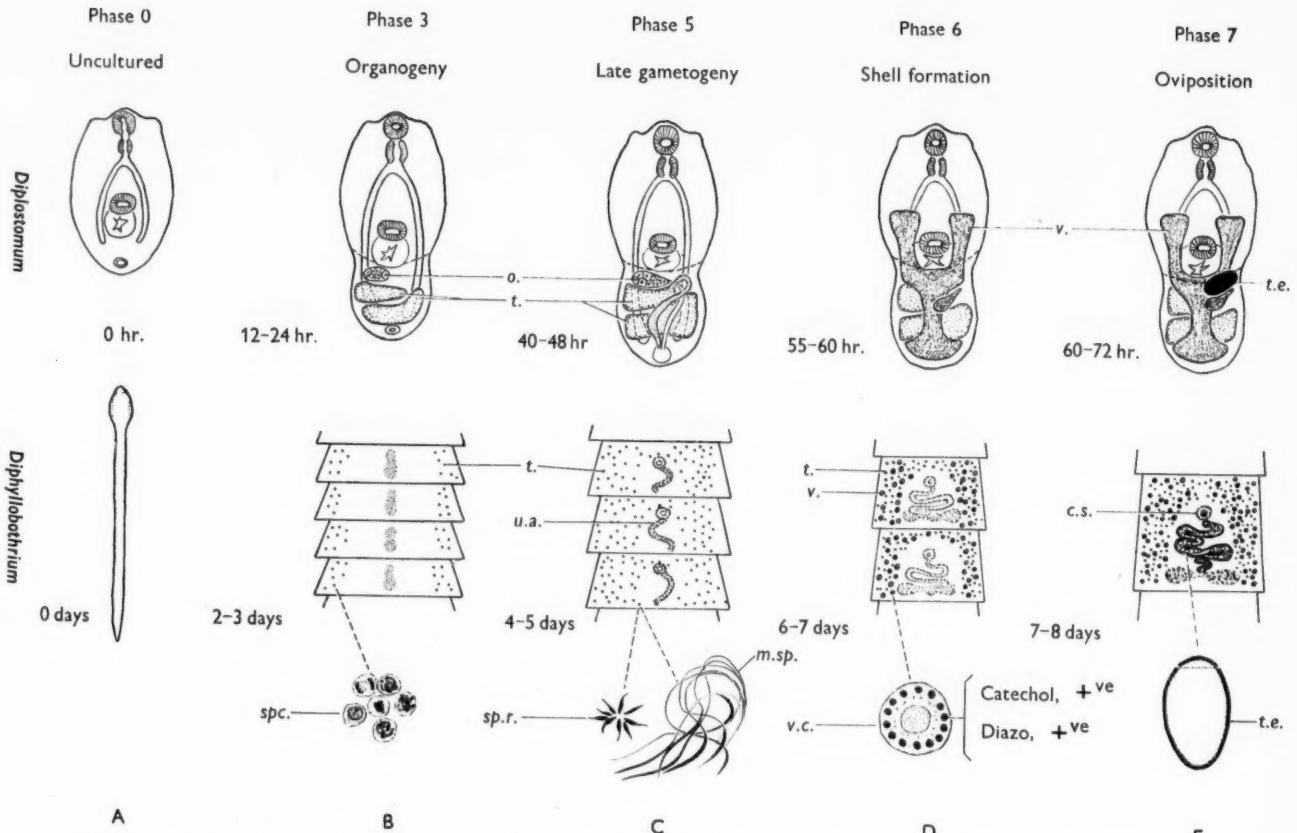

Fig. 1. Comparison of maturation in *Diphyllobothrium* and *Diplostomum in vivo*, showing the criteria used for the recognition of some of the phases. *c.s.* cirrus sac; *m.sp.* mature sperm; *o.* ovary; *spc.* spermatocyte; *sp.r.* sperm 'rosettes'; *t.* testes; *t.e.* tanned egg; *u.a.* uterine anlagen; *v.* vitellaria; *v.c.* vitelline cell.

extremely difficult, and the problem has not been efficiently solved for any tissue (Lushbaugh, 1956). The colchicine method as used here provides only a rough approximation of this activity. The growth of the tissues concerned, however, is virtually of an 'all or none' nature so that the method is adequate to enable a distinction between growing and non-growing tissue to be clearly made.

Method. Details of the method originally developed for *Diphyllobothrium* have been given by Smyth (1956*a*). After 24 hr. culture in the medium tested, larvae are further incubated in Tyrode's solution + 10^{-4} colchicine (sterilized by Seitz filtration) for a standard time, found by experiment to give a number of mitoses large enough to give an indication of activity. Treatment of 4 hr. for *Diplostomum* and 5 hr. for *Diphyllobothrium* were found to be satisfactory. Following colchicine treatment worms are treated with aceto-orcein and squashes prepared.

The time of treatment with aceto-orcein varies with the size of the organism. Exceptionally small trematodes such as *Diplostomum* stain and squash satisfactorily within 2–5 min. at room temperatures. Cestode material generally requires more prolonged staining; 40 min. at 37–40° C. is recommended. Squashes prepared in this way have only a life of a few hours unless the edges are sealed. We have found the coloured cellulose preparation used for correcting duplicator stencils excellent for this purpose and preparations so sealed are usable for 24 hr., and often several days.

Results. In practice, this has been found to be one of the most useful criteria for evaluating the suitability of culture media and conditions. It has the advantage that an evaluation can be arrived at within 24 hr. cultivation, after which time, depending on the result of the mitosis count obtained, experiments may be abandoned or continued.

Diphyllobothrium. The mitosis level in the rat host is of the order of 160–360 after 24 hr. culture (Table 1). In non-nutrient media, such as Tyrode's solution, counts as high as 110 are occasionally obtained although most figures are considerably lower (Smyth, 1956*a*). The fact that growth—as adjudged by mitosis—can occur at all in non-nutrient media is related to the presence of abundant larval food reserves, particularly glycogen (Smyth, 1947; Markov, 1943). The amount of growth possible in individual larvae will thus fluctuate with the reserve food materials available. During the first 12 hr. in such non-nutrient media counts over 300 may be obtained, but these drop to zero after 24–72 hr. culture when somatic mitosis virtually ceases, and degeneration commences. In the preliminary experiments, a large variety of nutrient media were used in attempts to obtain mitosis counts of a high order. Most of the media employed were saline media enriched with serum, glucose, yeast extracts, etc. None of these media gave mitosis counts approaching the 200 level.

Recently, by culturing excised fragments of *Diphyllobothrium* instead of whole plerocercoids and using highly concentrated nutrient media (embryo extract) counts of over 700 have been obtained, and in some instances the number of mitoses have been uncountable. In this medium, developmental stages up to spermatogenesis have been obtained (Smyth, in preparation). Results of evaluation of different media by this method are shown in Table 1.

Comparison of maturation in *Diphyllobothrium* and *Diplostomum in vivo*, showing the criteria used for the recognition of some of the phases. *c.s.* cirrus sac; *m.sp.* mature sperm; *o.* ovary; *spc.* spermatocyte; *sp.r.* sperm 'rosettes'; *t.* testes; *t.e.* tanned egg; *u.a.* uterine anlagen; *v.* vitellaria; *v.c.* vitelline cell.

Diplostomum. After 24 hr. in the duck intestine larvae show 150–200 inhibited mitoses per fluke (Bell & Hopkins, 1956). This number was taken as the 'standard' and medium and culture conditions altered until activity of a similar order was achieved *in vitro*. Results in material cultured *in vitro* were so striking that only an approximate threshold figure was required. Thus, in non-nutrient saline, virtually no mitoses (0–6) occur, although periods of survival up to 6 days are possible (Bell & Hopkins, 1956). In more recent experiments (Bell & Smyth, 1957) using complex viscous media, such as those containing egg-yolk, counts of 50–100 mitoses and improved development up to late spermatogenesis were obtained.

Table 1. *Evaluation of culture medium using mitosis as a criterion*

Organism	Medium	Mitoses after 24 hr. culture*	Prognosis	Maximum development achieved: Testis	Ovary	Cirrus	Uterus	Vitellaria	Eggs
Diphyllobothrium	Salines	100	Poor	–	–	–	–	–	–
	Serum	50	Poor	–	–	–	–	–	–
	Dilute embryo extract	50–130	Fair	–	–	–	+	–	–
	Conc. embryo extract	> 700	Excellent	+	?	+	+	–	–
	Rat (control)	160–360	Normal	+	+	+	+	+	+
Diplostomum	Salines	0–5	Poor	–	–	–	–	–	–
	Yolk	50–100	Fair	+	+	+	+	–	–
	Yolk and albumen	> 200	Excellent	+	+	+	+	+†	+†
	Duck control	> 150	Normal	+	+	+	+	+	+

* Counts made after treatment with colchicine for 4 hr. (*Diplostomum*) and 5 hr. (*Diphyllobothrium*).
† Developed abnormally.

Further improvement of the medium by the addition of albumen, together with alteration in the culture conditions by the introduction of discontinuous shaking to simulate intestinal movement, increased the mitosis figure to approximately 200 and organisms cultured under these conditions ultimately produced active spermatozoa and eggs. Although the latter proved to be abnormal (see p. 141), high initial counts, approaching those found in organisms matured *in vivo*, again served as excellent criteria for predicting successful early maturation stages.

Phase 2. Segmentation or body shaping

This phase occurs more clearly in developing cestodes and has no exactly comparable stage in trematodes.

Diphyllobothrium. In *Diphyllobothrium* it is heralded by the appearance of faint bands across the strobila. In living specimens early segmentation cannot be diagnosed accurately, but it may be detected in aceto-orcein preparations mounted without squashing. *In vitro*, in media giving low mitosis counts such as serum—even after 7–10 days' cultivation—segmentation never occurs. In media in which high mitosis counts were obtained, however, segmentation occurs on the second day, as in specimens matured *in vivo*. Segmentation is thus a useful general, though somewhat imprecise, criterion; its non-appearance within 2–3 days' cultivation may be taken as indicative of unsatisfactory culture conditions.

Diplostomum. The external changes which occur in trematodes are not as striking as in cestodes. In *Diplostomum* during the period 24–48 hr. the oval outline of the body is replaced by the bilobed condition (Fig. 1 B) typical of the adult strigeid; the posterior region, at first small, grows in size during subsequent maturation, and the edges become foliaceous. This change in body shape, although recognizable by an experienced observer, is not sufficiently precise to be used as a satisfactory criterion.

Phase 3. *Organogeny*

Organogeny, as a criterion, is in general a more satisfactory one in cestodes than in trematodes. The detection of organ primordia can best be carried out in aceto-orcein squashes, aceto-orcein whole mounts or well-differentiated whole mounts prepared by any of the usual carmine or haematoxylin methods.

Diphyllobothrium. Development taking place after the third day may be estimated accurately by the macroscopic condition of female and male genitalia in stained preparations. Although genital primordia are just visible *in vivo* on the second day, they do not become unmistakably clear until the third day. The tubular organs appear as an accumulation of cells in the central region, the whole gradually assuming a dumb-bell shape, the primordia of the testes appearing at the same time (Fig. 1 B). By the fifth day, differentiation into cirrus pouch and uterus is complete and these organs become recognizably tubular (Fig. 1 C). By the sixth day, the uterus assumes its characteristic coiled shape and the uterine and cirrus opening can be recognized (Fig. 1 D).

Diplostomum. Early genital primordia are not so well defined as in *Diphyllobothrium* owing to the small size of the organism; their appearance does not become sufficiently definite until 48 hr. *in vivo*, by which time the presence of mature spermatozoa serves as a more reliable and more easily recognizable criterion.

Phase 4. *Early gametogeny*

In both organisms, detection of the various stages in spermatogenesis formed one of the most satisfactory of all the criteria used. These are readily seen in aceto-orcein squashes without previous treatment in colchicine.

Criteria have not been developed for the various stages of oogenesis. Maturing early ova give no characteristic histochemical reactions, and in general present no criteria for their ready recognition. The fully mature ovary is recognizable in *Diplostomum* in squashes, by its general form and position, individual ova having characteristically large nuclei and nucleoli. The mature ovary in *Diphyllobothrium* is difficult to locate in squashes.

Diphyllobothrium. In specimens matured *in vivo*, the earliest testes primordia appear on the third day as cells collected into typical spherical groups which may show intensive mitosis (*spc*. Fig. 1 B). During the fourth to fifth day spermatid morula become differentiated, and these appear in aceto-orcein squashes as the well-known 'rosettes' (*sp.r*. Fig. 1 C) of elongated nuclei which later become somewhat bent back on themselves to form the 'comma' stages.

Diplostomum. The testes occur as large discrete bodies and not as scattered

groups of cells. The stages of spermatogenesis are readily recognizable in squashes and follow the same pattern as in *Diphyllobothrium*. Early spermatogenesis is accompanied by intensive mitosis and probably the majority of observed mitoses in Phase 1 occur in the testes. The 'rosette' stage is especially clear, and together with the 'comma stage' form unmistakable criteria as to the progress of development.

Phase 5. Late gametogeny

This phase is characterized by maturation and release of sexual products; the presence of mature spermatozoa serving as one of the most easily recognizable criteria of the whole developmental pattern. They may be detected in freshly teased worms or in aceto-orcein squashes. The mere presence of spermatozoa is not sufficient to indicate 'normal' development; spermatozoa must be morphologically normal, show movement, and occur in quantities comparable to those in worms matured *in vivo*. All combinations of abnormalities are possible: morphologically abnormal sperm morula showing no activity; morphologically normal spermatozoa showing no activity; morphologically normal spermatozoa showing activity, but present in numbers less than those found in *in vivo* matured specimens.

Diphyllobothrium. This criterion has been of considerable value and *in vitro* development has been achieved up to the appearance of mature spermatozoa within the same time as *in vivo*—(5–6 days) (Smyth, in preparation); the spermatozoa, however, cannot be considered normal for they were neither active nor present in quantity.

Diplostomum. The time taken for spermatozoa to appear, their quantity and activity have similarly served as useful criteria in assessing development. In early experiments, gradual improvement in culture conditions resulted in spermatozoa being produced *in vitro* after 66 hr. culture compared with 40–48 hr. required *in vivo*; the quantity produced was smaller than *in vivo*. Further improvement in techniques resulted in a shortening of the maturation time to 40 hr. and an increase in the numbers developed, until eventually no difference could be observed in the activity or number of spermatozoa matured *in vivo* or *in vitro*.

Phase 6. Egg-shell formation and vitellogenesis

Mechanism of egg formation. As the male and female gametes near maturity, the rudiments of the vitellaria make their appearance as groups of cells which show at first no recognizable characteristics. As maturation proceeds, these cells increase in size and the precursors of the shell material appear in the cytoplasm as small globules. When the vitelline cells become fully mature, the egg-shell material is in the form of numerous large globules (Fig. 1 D) just visible in fresh preparations and which, in some helminths, have a yellow tinge. The mechanism of egg-shell formation follows the pattern typical of the majority of trematodes and pseudophyllidean cestodes (Dawes, 1940; Kouri & Nauss, 1938; Stephenson, 1947; Smyth, 1954*b*, 1956*b*; Gonnert, 1955). A fertilized ovum passes into the lower uterus region where it meets the 'vitelline' cells. Approximately at Mehlis' gland region, the vitelline cells release their shell globules which run together to form a semi-liquid

shell which becomes moulded into an egg shape; the moulded egg passes down the uterus and its shell hardens and 'tans'. The remains of the vitelline cells serve as yolk for the developing egg. All recent work (Stephenson, 1947; Romanini, 1947; Vialli, 1950; Smyth, 1954*b*, 1956*b*; Gonnert, 1955; Johri & Smyth, 1956) suggests that the egg-shell in the majority of species in these groups is composed of sclerotin, a quinone-tanned structural protein widespread in the animal kingdom, although several exceptions are known. The basic constituents of sclerotin are protein, phenolic substances and polyphenol oxidase, from which it follows that the egg-shell material in the vitelline cells may be traced by means of histochemical methods for detecting these substances.

Suitable histochemical methods for sections or whole mounts have been described in detail (Smyth, 1954*b*, 1956*b*; Johri & Smyth, 1956). The choice of methods will depend on the nature of the material and the reagents available.

Diazo technique for egg-shell precursors. One of the most useful histochemical techniques is that incorporating the use of stable diazotates which link readily with phenolic materials giving characteristic colours. The earliest appearance and subsequent fate of the egg-shell precursors may be detected by means of this method. It has the advantage that it may usually be applied to whole mount preparations with consequent saving of time. Since the vitelline globules are small when they first appear, it is important that specimens to which this test is applied should be flattened to the limit (without bursting) before and during fixation. 70% ethanol, though poor as a cytological fixative, is the best general fixative for most of these tests which are concerned only with the presence of substances within a cell, and not with the precise distribution of that substance within the cell. Details of the method have been given by Johri & Smyth (1956), but are repeated in outline below.

(1) Flatten and fix in 70% ethanol 2–24 hr.

(2) Wash in H_2O—15 min.

(3) Place in 1% Fast Red salt B* (freshly prepared and filtered) in a watch-glass and observe under a low-power binocular. In small trematodes, the phenolic shell precursors in the vitelline cells become red within ¼–3 min. Larger trematodes or thick cestodes may require up to 15 min.

(4) Wash in H_2O—15 min.

(5) Stain in Gower's carmine 1–3 hr. (optional); differentiate in acid alcohol.

(6) Dehydrate, clear and mount.

The colour developed in the egg-shell precursors depends on the nature of the particular phenolic compounds present. It ranges from yellow or orange to orange-red or bright red.

Diphyllobothrium. In *Diphyllobothrium*, and in other pseudophyllidean cestodes examined, the diazo reaction given by the vitelline cells is amber or yellow-orange and never red or orange-red as in trematodes. This is a phenomenon not properly understood (Smyth, 1956*b*), but is clearly related to the nature of the phenolic materials present. *In vivo*, *Diphyllobothrium* becomes diazo + ve on the sixth day,

* G. T. Gurr, London, S.W. 6.

but developmental stages with diazo + ve vitelline cells have not been obtained *in vitro* to date.

Diplostomum. In specimens matured *in vivo*, the vitellaria become diazo + ve at 55 hr., some 5 hr. before fully formed eggs appear. The colour reaction is red or orange-red, the vitellaria standing out brilliantly against an almost colourless background (Fig. 2A). In early attempts to mature these organisms *in vitro*, the

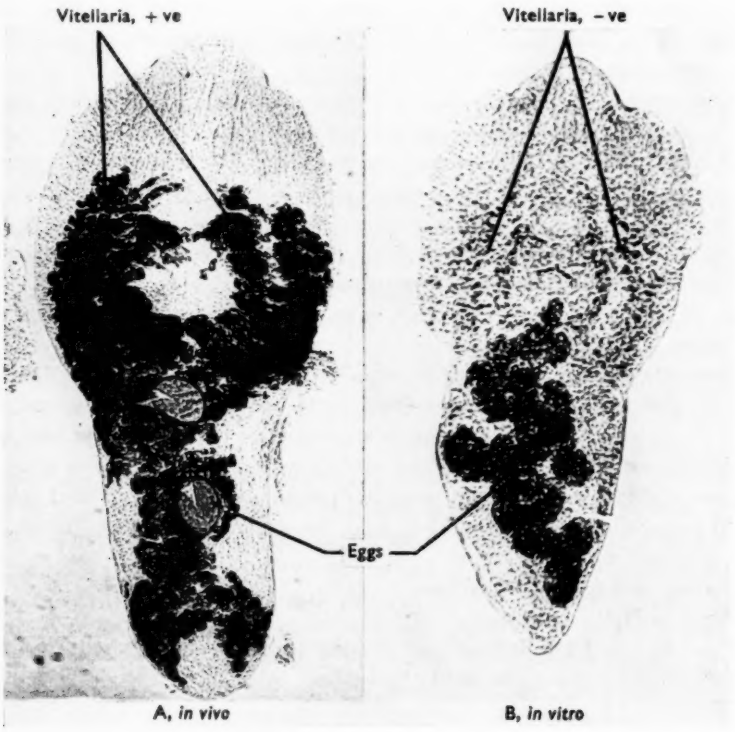

Fig. 2. Comparison of *Diplostomum phoxini* matured: A, *in vivo* (duck, 3 days) and B, *in vitro* (yolk, albumen, proline, hydroxyproline mixture, 5 days). Fixation 70%. Treated diazo reagent 3 min. Upgraded. Note the poorly developed outlines of the eggs and almost complete lack of diazo + ve material (egg-shell precursors) in the vitellaria of the *in vitro* specimen.

most advanced stage of maturation obtained showed partly formed vitellaria, which in contrast (Fig. 2B) gave only a yellow or weakly orange-yellow diazo reaction (Bell & Smyth, 1957).

This is strongly suggestive of an abnormal synthesis of the protein or phenolic precursors of the shell-material possibly due to a nutritional deficiency in the medium. The nature of this deficiency has not yet been determined. The vitelline cells have many properties similar to those of other quinone tanning systems such as that secreting the byssus of *Mytilus* (Smyth, 1954*b*; Brown, 1952) or the egg capsule of elasmobranchs (Brown, 1955), and since the protein concerned in these

systems is known from X-ray analysis to be collagen, it is possible that the protein of the trematode egg, and perhaps the pseudophyllidean cestode egg also, may likewise prove to belong to the collagen group of structural proteins. If this should prove to be the case, it is likely that the precursors of the egg-shell material are closely related to those required in other collagen-forming systems. It is suggestive that when the culture medium for *Diplostomum* is augmented by gelatine (i.e. a breakdown product of collagen-containing tissues) the vitelline cells although still poorly developed give a reddish-orange colour more nearly approaching that given by *in vivo* forms (Bell & Smyth, 1957). The effect of adding to the medium various amino-acids likely to be concerned in collagen synthesis is being investigated, but even in the best results there are marked differences between the vitellaria in the *in vivo* (Fig. 2A) and *in vitro* matured forms (Fig. 2B).

The argentaffin reaction. The argentaffin reaction is a non-specific one, and may be given by various reducing substances (Lillie, 1954; Tandler, 1955), but in the demonstrable absence of alternative substances in helminths, a positive reaction may tentatively be taken to indicate phenols, as only precursors of the shell material in the vitellaria have given a strongly positive reaction in all trematodes and pseudophyllidean cestodes studied (Smyth, 1954*b*, 1956*b*; Stephenson, 1947). Nevertheless, silver techniques are notoriously unreliable and results based on their use should be interpreted with caution. The method normally used in histochemistry is carried out on sections, but it may be adapted to whole mounts; vitellaria containing shell precursors react red to black depending on how long the reaction is permitted to proceed, and the concentration of silver nitrate used. In 5% $AgNO_3$ a positive result becomes evident within 1 hr., in 0·1% $AgNO_3$ 12 hr. is required. The technique for whole mounts is as follows:

(1) Fix in 5% formol—2–12 hr.

(2) Wash in running water 15 min.–1 hr.

(3) Place in 0·1% ammonical silver nitrate (add concentrated ammonia to 0·1% $AgNO_3$ drop by drop until precipitate dissolves), 12–24 hr. The reaction may be stopped when a recognizable colour has developed in the vitellaria.

(4) Wash in 1% ammonia—2 hr.

(5) Upgrade, clear and mount.

In *Diplostomum* and *Diphyllobothrium* the result closely parallels that with the diazo technique, but the method is unsuitable for permanent whole-mount preparations as the cytoplasm may take on a reddish yellow tinge on standing.

Alternative methods of detecting egg-shell precursors

There are a number of other methods of detecting egg-shell precursors, some of which may be used on whole mounts of helminths as well as sections, and serve as confirmatory evidence for the diazo reaction; others are too destructive or give colours cytologically unsatisfactory. The most useful of these methods is the catechol reaction for detecting polyphenol oxidase. This consists of incubating ethanol-fixed material in 0·1% catechol at 40° C., a yellow to orange-red coloration developing at the enzyme site. With trematodes, such as *Diplostomum*, the reddish-brown colour developed is nearly as brilliant as with the diazo technique. With

cestodes only an amber colour is developed. Full technical details of the method are given by Smyth (1945*b*) and Johri & Smyth (1956).

This technique has a particular advantage over the diazo technique in that it not only gives a colour reaction with the vitellaria but also with the completed egg-shell. Methods based on the presence of phenolic materials (e.g. diazo, argentaffin) gave either negative or only weakly positive reactions with finished egg-shells situated far up the uterus, as in this region the enzymatic oxidation of phenols to quinones has taken place, although shells just formed may give quite strong reactions. A residue of polyphenol oxidase, however, remains in the shell so that the formed eggs give a characteristic catechol reaction. This method has the advantage of demonstrating the position and shape of eggs in the uterus, and may be of value for the detection of abnormal uterine development.

Phase 7. *Oviposition*

General morphology. The oviposition phase is characterized by the appearance of fully formed eggs. If numerous, these may be detected in living material by gently compressing under a cover-glass. The colour of the egg-shell, its contours and size all serve as useful criteria when eggs of specimens matured *in vivo* and *in vitro* are compared. A more precise evaluation may, however, be obtained by fixing compressed specimens in 70 % ethanol and treating with catechol or diazo reagents, as in the previous section, when the eggs will become clear. In *Diphyllobothrium*, egg-shell material of any kind has not appeared in specimens matured *in vitro* and in *Diplostomum* only abnormally shaped eggs giving weak catechol and diazo reactions have been produced.

Testing egg embryonation. The final test of successful maturation, however, must be the fertility of the eggs and their subsequent development through the larval stages. In our general experience eggs from common forms such as *Diphyllobothrium latum* or *Fasciola hepatica* show a range of 80–90 % embryonation. It is relatively easy to test embryonation in these species on account of the large number of eggs available. With small trematodes—e.g. certain strigeids—the number of eggs present in the uterus at any one time may be only one to five, and the problem of testing embryonation without undue risk of loss or damage is more difficult.

A method found suitable for both small and large numbers of eggs is to place these on a cellophane disk (about 1½ in. diameter) in a watch-glass covered with about ⅛ in. water in a Petri dish, and incubate at 25° C. After 24 hr. at this temperature the eggs become attached to the surface of the cellophane; this prevents their loss when the water is being renewed daily, and facilitates transfer of the embryonated eggs for subsequent hatching and infection of copepods or molluscs, as the entire cellophane disk may be added to the culture of these hosts.

Criteria for early larval stages. In the present experiments, fertile eggs have not been obtained with either *Diphyllobothrium* or *Diplostomum*, but some previous results with *Schistocephalus* and *Ligula*, probably typical of pseudophyllidean development, are relevant here. In *Schistocephalus* the earliest experiments resulted in abnormal eggs showing no embryonation (Smyth, 1946). Environ-

mental conditions were later (1950) improved to permit the production of morphologically and cytologically normal eggs and, although such eggs embryonated, the coracidia produced were undersized, unhatchable, and believed to be haploid in genetic constitution, owing to failure of fertilization. This was confirmed when culture conditions were further improved by the introduction of the cellulose tube technique (Smyth, 1954*a*) to simulate the compression of intestinal surfaces and assure fertilization; under these conditions morphologically normal, full-sized hatchable coracidia were then obtained.

Thus caution is necessary even in the use of embryonation as a criterion, and differences in size, inability to hatch or other abnormalities should be carefully watched for.

Criteria for the maintenance of the adult condition

Once the fully mature condition is reached, it is important to develop criteria to determine whether or not the metabolism is being maintained at the adult level. This is similarly true when adult worms are initially used in culture experiments.

A continual supply of eggs equivalent in number and fertility to those produced *in vivo* would, of course, supply a precise criterion. One difficulty which arises here is that although the total number of eggs produced may be known, testing of fertility (by embryonation) takes at least 7 days at 25° C, so that immediate evaluation of the condition of the eggs cannot be arrived at. Other criteria of a cytological or histochemical nature suffer from a further disadvantage in that cultured specimens must be destroyed by fixation before an evaluation can be arrived at. Notwithstanding these disadvantages, however, fertility and absence of cytological abnormalities are the most satisfactory criteria at present available.

Cytological methods may give rapid evaluation. Work on *Schistocephalus* (Smyth, 1946, 1952), *Ligula* (Smyth, 1949) and *Fasciola* (Clegg, 1957) has shown that the testes are extremely sensitive to unfavourable cultural conditions. In *Schistocephalus*, an unfavourable pH or temperature of cultivation is reflected in the appearance in the testes of 'giant cells'—abnormal spermatocyte or spermatic morula. These abnormalities may be detected in sections and often in aceto-orcein squashes although they show a tendency to burst with the latter technique.

In *Fasciola*, Clegg (1957) has shown that some cytological abnormalities can be detected after only 3 hr. cultivation in non-nutrient media such as Hédon–Fleig, and that there is a steady increase in the percentage abnormality with time. In order to avoid tedious sectioning, this worker has developed a technique of teasing the testes in saline, centrifuging and making smears. He has found that both the appearance of 'giant' cells and the relative number of morula showing metaphases can serve as criteria.

A combination of cytological examination and testing of egg number and fertility should provide a reasonably clear indication as to whether or not the metabolic activities of an organism are being maintained at the 'normal' adult level. This is especially important in biochemical metabolic studies in which data of a fundamental nature are being determined.

DISCUSSION

It has been shown that the development of the cestode *Diphyllobothrium* and the trematode *Diplostomum* have certain features in common which may serve as criteria for assessing maturation. Early workers (including the senior writer) have used survival as an initial criterion for evaluating the success of helminth culture methods, but it is becoming increasingly clear that survival as a criterion, unrelated to the cytological and morphological condition of the organism, is of little value except at the very onset of *in vitro* work. Its use may, in fact, produce grossly misleading pictures. Thus the cestode *Schistocephalus* matures *in vivo* in 36 hr., and maturation is obtained *in vitro* in the same time under suitable conditions (Smyth, 1954*a*). After 36 hr. culture a worm produces quantities of eggs and continues to do so for 2–4 days, at the end of which time, having used up its endogenous reserves, it dies. On the other hand, if larvae are cultured in a small quantity of poorly buffered medium the acid excretory products released so inhibit the metabolism that although worms continue to show activity, little tissue differentiation takes place. 'Survival' under these unsuitable conditions may be as long as 10 days—more than twice its 'normal' longevity *in vivo*.

Thus, increased survival is not *per se* indicative of improved *in vitro* conditions and may, as with *Schistocephalus*, point to exactly the opposite. The same pattern is followed in *Diphyllobothrium* and *Diplostomum*, the larvae of which show activity for 7 days or more (Smyth, 1947; Bell & Hopkins, 1956) in certain media without undergoing development. Application of precise criteria would eliminate such methods as unsuitable after 24 hr. incubation. That survival is of little value as a criterion is still not generally appreciated by some workers, and Rohrbacher (1957) has recently estimated the sucess of *in vitro* experiments with *Fasciola* on survival grounds alone. With this organism Clegg (1957) has shown that cytological abnormalities rapidly develop in unsuitable media.

Criteria selected here for assessing development have been essentially cell-division, spermatogenesis, egg-shell formation and fertility. These represent one set of criteria which have been found convenient, but alternative or additional criteria may emerge in subsequent work. A satisfactory criterion should be precise and readily and rapidly identifiable. The more precise and exact it is, the more readily can slight changes in *in vitro* development be detected. It is important to detect a change in the developmental pattern as soon as possible, so that prolonged periods of cultivation should not be necessary before the growth-producing powers of a medium are assessed. Used singly or in combination, the criteria cited here can usually detect beneficial or detrimental changes within 24–48 hr. at 40° C. Detection of mitosis after colchicine inhibition has proved the most efficient in this respect, and, with both *Diphyllobothrium* and *Diplostomum*, cultures which fail to show a high mitosis rate after 24 hr. may be abandoned. This results in an immense saving of time, and enables a rapid screening of media and culture conditions to be made.

Results with colchicine must, however, be interpreted with caution. A high mitotic count indicates a high rate of cell multiplication and serves as an excellent

criterion for that particular phase of development—but that phase only; it does not necessarily imply that the medium or cultural conditions which were found suitable for maintaining rapid nuclei multiplication are necessarily suitable for the later phases, some of which are synthetic phases involving materials other than those concerned in nuclear synthesis.

Results with *Diphyllobothrium* fragments (Smyth, in preparation) illustrate this point. In concentrated embryo extract the mitotic rate is as high as, or higher than, *in vivo*, and after 24–48 hr. culture there is an enormous increase in the number of

Table 2. *Criteria recommended for the recognition of developmental phases in the trematode* Diplostomum *and the cestode* Diphyllobothrium

Phase	Time in host: *Diplostomum* (hr.)	Time in host: *Diphyllobothrium* (days)	Criterion recommended	Method of detection
(1) Cell multiplication	0–24	0–1	Mitoses counts	Aceto-orcein squashes after colchicine treatment
(2) Segmentation or body shaping	24–48	1–2	Division into proglottides (*Diphyllobothrium*) or regions (*Diplostomum*)	Direct observation on living material or aceto-orcein squashes
(3) Organogeny	12–24	2–3	Appearance of uterus and testes primordia	Squashes or whole mounts
(4) Early gametogeny	36–40	4–5	Appearance of 'rosette' and 'comma' stages in spermatogenesis	Squashes
(5) Late gametogeny	40–48	5–6	Appearance of mature spermatozoa	Squashes or unstained teases
(6) Egg-shell formation and vitellogenesis	55–60	6–7	Presence of egg-shell precursors in 'vitelline' cells	Histochemical tests on whole specimens. Diazo +ve, catechol +ve.
(7) Oviposition	60–72	7–8	Appearance of fully formed egg	Direct observations on living material or catechol-treated whole mounts

embryonic cells. Such embryonic cells consist essentially of nuclei with little surrounding cytoplasm. The only organ systems which can develop from such cells without additional nutritive materials being absorbed are those which require mainly reorganization without intensive synthesis of new cytoplasmic materials—e.g. spermatozoa and, to some extent, early tubular genitalia. Thus, a high mitotic count can be used to predict that the medium will be suitable for development up to spermatogenesis. Such a hypothesis is generally borne out by the results in Table 2.

In *Diphyllobothrium* the later phases of development, such as egg-shell formation, involve cytoplasmic synthesis of great quantities of protein pointing to the necessity of food requirements of a high order. The failure of this stage to develop *in vitro* in spite of high initial mitotic counts indicates that, although the culture medium used can satisfy the nutritional requirements up to spermatogenesis, it cannot meet the enormous demands of the egg-shell production stages.

The question of time sequence is of particular importance in developmental studies, and in this respect each criterion is of double value. To satisfy a criterion, a certain developmental feature must not only appear, but it must do so within

the same time range as it does *in vivo*. It is well known that with some helminths the maturation time varies with the host so that precise definition of this may not always be possible. The same organism may behave differently, mature slower or produce fewer eggs in different hosts. Nevertheless, a range of *in vivo* patterns can be established and attempts made to achieve *in vitro* development within those limits; serious time discrepancies from the developmental pattern may be taken to indicate abnormal development. Such marked differences between *in vivo* and *in vitro* developmental times are characteristic of early developmental studies. Thus, Ferguson (1940) matured *Posthodiplostomum minimum in vitro* and found that maturation as adjudged by the appearance of eggs required 4 days compared with 35–40 hr. *in vivo*. Weinstein & Jones (1956) in their recent interesting work on the development of *Nippostrongylus muris in vitro* similarly found that the fifth-stage larvae took three to four times longer to develop *in vitro* than *in vivo*.

In pseudophyllidean cestodes, the most satisfactory criteria are mitosis, segmentation, appearance of testes primordia, spermatogenesis, maturation of vitellaria and egg-formation, in that order. Use of these criteria have led to the development of plerocercoids up to spermatogenesis and have revealed the limitations of the culture methods at present employed. Some of these methods—especially those covering the early stages of development and early spermatogenesis—may be directly applicable to the cyclophyllidean cestodes whose early growth phases have much in common. The so-called egg-shell in this group differs markedly from the tanned egg-shell in the pseudophyllidean cestodes as does the character of the vitellaria (Smyth, 1951, 1956*b*; Johri, 1957), so that criteria involving the use of diazo or catechol reactions are no longer applicable, and alternative criteria specific for this group will need to be developed.

In trematodes the most satisfactory criteria have proved to be mitosis, spermatogenesis, maturation of vitellaria and egg-shell formation in that order. In connexion with the use of the latter criterion, it must be stressed that although the trematode used in the present study, and probably the majority of trematodes, form a quinone-tanned egg, it has been found that the egg-shell of several forms (Smyth & Howie, unpublished work), while giving a reaction with the diazo reagent, gives a negative test for polyphenol oxidase with catechol. This suggests the existence in trematodes of an alternative enzyme system for the stabilization of egg-shell protein. Failure to give a positive catechol reaction must thus be interpreted cautiously and confirmatory tests carried out with the diazo reagent. We have failed, so far, to find a trematode whose egg-shell material does not give a positive diazo reaction.

SUMMARY

1. Maturation of the plerocercoid of the pseudophyllidean cestode *Diphyllobothrium* sp. and the metacercaria of the strigeid trematode *Diplostomum phoxini* follow a similar pattern, which may be divided into the following phases: (1) cell multiplication; (2) segmentation or body shaping; (3) organogeny; (4) early gametogeny; (5) late gametogeny; (6) egg-shell formation and vitellogenesis; (7) oviposition.

2. Cytological or histochemical criteria have been developed by means of which each developmental phase may be recognized. Development *in vitro* may be considered comparable with that occurring *in vivo* if the same phases are completed with the same incubation time and without the appearance of cytological abnormalities.

3. The cell multiplication phase is characterized by high mitotic activity. This activity *in vitro* can be evaluated by incubating larvae after 24 hr. culture for a standard time (5 hr. *Diphyllobothrium*; 4 hr. *Diplostomum*) in 10^{-4} colchicine and comparing the number of mitoses seen in a defined area of an aceto-orcein squash with those found in tissue matured *in vivo* for the same period. Use of this criterion enables the growth-producing properties of a medium to be assessed after 24 hr. culture.

4. Phases 2–4 are characterized by the appearance of the tubular genitalia, the rudiments of which are readily recognized in aceto-orcein squashes or whole mount preparations.

5. The presence of spermatids and mature spermatozoa (phases 4–5) may be detected in aceto-orcein squashes or in fresh tissue teases. The quantity and activity of spermatozoa also serve as important criteria.

6. Recognition of phase 6, egg-shell formation, is carried out by means of diazo reagents which give orange or orange-red reactions with the phenolic egg-shell precursors in the vitellaria, as seen in whole mount preparations. Abnormal egg-shell development is reflected in the failure of vitellaria to give a normal diazo reaction.

7. The appearance of eggs which are macroscopically normal constitutes the final maturation phase. Early detection of eggs (especially abnormal ones) is facilitated by the use of the diazo reaction or the catechol test for polyphenol oxidase.

8. Embryonation and hatching of eggs to produce morphologically normal infective coracidia or miracidia serve as a final criteria.

9. Criteria for the maintenance of the adult condition are also considered.

REFERENCES

Archer, D. M. & Hopkins, C. A. (1957). Studies on cestode metabolism. III. The growth pattern of *Diphyllobothrium* sp. (Cestode: Pseudophyllidea) in a definitive host. *Exp. Parasitol.* (in the Press).

Bell, E. J. & Hopkins, C. A. (1956). The development of *Diplostomum phoxini* (Strigeida, Trematoda). *Ann. Trop. Med. Parasit.* **50**, 275–82.

Bell, E. J. & Smyth, J. D. (1957). Axenic development of *Diplostomum phoxini* (Strigeida, Trematoda). Unpublished work.

Brown, C. H. (1952). Some structural proteins of *Mytilus edulis*. *Quart. J. Micr. Sci.* **93**, 487–503.

Brown, C. H. (1955). Egg-capsule protein of Selachians and trout. *Quart. J. Micr. Sci.* **96**, 483–8.

Clegg, J. (1957). Studies on the maintenance of *Fasciola hepatica in vitro*. Ph.D. Thesis. Univ. of London.

Dawes, B. (1940). *The Trematoda*. Cambridge University Press.

Ferguson, M. S. (1940). Excystment and sterilization of metacercaria of the avian strigeid trematode, *Posthodiplostomum minimum* and their development into adult worms in sterile cultures. *J. Parasit.* **26**, 359–72.

GONNERT, R. (1955). Schistosomiasis-Studien. II. Über die Eibildung bei *Schistosoma mansoni* und das Schicksal der Eier im Wirtsorganismus. *Z. Tropenmed. u Parasit.* **6**, 33–52.

JOHRI, L. N. (1957). A morphological and histochemical study of egg-shell formation in a cyclophyllidean cestode. *Parasitology*, **47**, 21–29

JOHRI, L. N. & SMYTH, J. D. (1956). A histochemical approach to the study of helminth morphology. *Parasitology*, **46**, 107–116.

KOURI, P. & NAUSS, R. W. (1938). Formation of the egg-shell in *Fasciola hepatica* as demonstrated by histological methods. *J. Parasit.* **24**, 291–310.

KUHLOW, F. (1953). Über die Entwicklung und Anatomie von *Diphyllobothrium dendriticum* Nitzsch 1824. *Z. Parasitenk.* **16**, 1–35.

LILLIE, R. D. 1954. Argentaffin and Schiff reactions after periodic acid oxidation and aldehyde blocking reactions. *J. Histochem. Cytochem.* **2**, 123–36.

LUSHBAUGH, C. C. (1956). Morphological methods of determining doubling times. *J. Histochem. Cytochem.* **4**, 499–507.

MARKOV, G. S. (1943). The dynamic of reserve nutritive substances in parasitic worms in artificial media. *Zool. Zh.* **22**, 3–18.

RAUSCH, R. L. (1956). Studies on the helminth fauna of Alaska. XXVIII. The description and occurrence of *Diphyllobothrium dalliae* n.sp. (Cestoda). *Trans. Amer. Micr. Soc.* **75**, 180–7.

ROHRBACHER, G. H. (1957). Observations on the survival *in vitro* of bacteria-free adult common liver flukes, *Fasciola hepatica* Linn., 1758. *J. Parasit.* **43**, 9–18.

ROMANINI, M. G. (1947). Contributo alla conoscenza istochimica dei vitellogeni di distoma hepaticum. *Monit. zool. ital.* **56**, 3–6.

SMYTH, J. D. (1946). Studies on tapeworm physiology. I. Cultivation of *Schistocephalus solidus in vitro*. *J. Exp. Biol.* **23**, 47–70.

SMYTH, J. D. (1947). Studies on tapeworm physiology. III. Aseptic cultivation of larval *Diphyllobothriidae in vitro*. *J. Exp. Biol.* **24**, 374–86.

SMYTH, J. D. (1949). Studies on tapeworm physiology. IV. Further observations on the development of *Ligula intestinalis in vitro*. *J. Exp. Biol.* **26**, 1–14.

SMYTH, J. D. (1950). Studies on tapeworm physiology. V. Further observations on the maturation of *Schistocephalus solidus* (*Diphyllobothriidae*) under sterile conditions *in vitro*. *J. Parasit.* **36**, 371–83.

SMYTH, J. D. (1951). Egg-shell formation in trematodes and cestodes as demonstrated by the methyl or malachite green techniques. *Nature, Lond.*, **168**, 322–3.

SMYTH, J. D. (1952). Studies on tapeworm physiology. VI. The effect of temperature on the maturation of *Schistocephalus solidus in vitro*. *J. Exp. Biol.* **29**, 304–9.

SMYTH, J. D. (1954*a*). Studies on tapeworm physiology. VII. Fertilization of *Schistocephalus solidus in vitro*. *Exp. Parasit.* **3**, 64–71.

SMYTH, J. D. (1954*b*). A technique for the histochemical demonstration of polyphenol oxidase and its application to egg-shell formation in helminths and byssus formation in *Mytilus*. *Quart. J. Micr. Sci.* **95**, 139–52.

SMYTH, J. D. (1956*a*). Studies on tapeworm physiology. VIII. Occurrence of somatic mitosis in *Diphyllobothrium* spp. and its use as a criterion for assessing growth *in vitro*. *Exp. Parasit.* **5**, 260–70.

SMYTH, J. D. (1956*b*). Studies on tapeworm physiology. IX. A histochemical study of egg-shell formation in *Schistocephalus solidus* (Pseudophyllidea). *Exp. Parasit.* **5**, 519–40.

SMYTH, J. D. (1957). In preparation.

SMYTH, J. D. & HOWIE, D. I. D. (1957). Unpublished work.

STEPHENSON, W. (1947). Physiological and histochemical observations on the adult liver fluke, *Fasciola hepatica*. L. III. Egg-shell formation. *Parasitology*, **38**, 128–139.

TANDLER, C. J. (1955). The reaction of nuclei with ammonacal silver nitrate in the darkness. *J. Histochem. Cytochem.* **3**, 196–202.

VIALLI, M. (1950). Fenoli e tannazione chinonica delle proteine. *Monit. zool. ital.* **58**, 83–87.

WEINSTEIN, P. P. & JONES, M. F. (1956). The *in vitro* cultivation of *Nippostrongylus muris* to the adult stage. *J. Parasit.* **42**, 215–36.

(*MS. received for publication* 16. v. 1957.—Ed.)

THE OXYGEN UPTAKE OF *TRYPANOSOMA LEWISI* AND *TRYPANOSOMA EQUIPERDUM*, WITH ESPECIAL REFERENCE TO OXYGEN CONSUMPTION IN THE PRESENCE OF AMINO-ACIDS

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(With 7 Figures in the Text)

CONTENTS

PAGE

I. Introduction 149

II. Materials and methods 150

III. Oxygen uptake in the absence of substrate 151
(1) *Trypanosoma lewisi* 151
(2) *Trypanosoma equiperdum* 152

IV. Effect of single substrates on oxygen uptake 153
(1) *Trypanosoma lewisi* 153
(*a*) Comparison of adult and reproductive phases 153
(*b*) Oxygen uptake with amino-acids and other compounds . . . 155
(2) *Trypanosoma equiperdum* 156
(*a*) Oxygen uptake with glucose and glycerol 156
(*b*) Oxygen uptake with amino-acids and other compounds . . . 158

V. The evolution of ammonia during the metabolism of glutamine . . . 159
(1) Liberation of ammonia from glutamine 159
(2) Liberation of ammonia by *Trypanosoma lewisi* 160

VI. Discussion 162

VII. Summary 162

References 163

I. INTRODUCTION

Experiments on the metabolism of the blood-stream form of trypanosomes have shown that there are fundamental differences between *Trypanosoma lewisi* and the *T. evansi-brucei* group of trypanosomes. References to early work are given by von Brand (1951) and to later work by Ryley (1956). However, most of the experiments on *T. lewisi* have been carried out in the absence of serum, while many of the experiments on the *T. evansi-brucei* group have been carried out in the presence of serum or glucose.

The purpose of the present investigation was to compare the effect of amino-acids on the oxygen consumption of blood-stream forms of *T. lewisi* and *T. equiperdum* in buffered salt solutions in the absence of serum.

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II. MATERIALS AND METHODS

The strain of *T. lewisi* was the same as that described by Ryley (1951), but it has changed its character in that the reproductive phase now ends about 5 days after inoculation and the trypanosomes have usually disappeared from the blood by 10–12 days. The strain is maintained in hooded or albino rats by intraperitoneal inoculation once a week with infected blood in citrate saline solution.

The strain of *T. equiperdum* was received from the National Institute for Medical Research in 1955 and has been maintained in mice since then.

The trypanosomes were obtained from infected rats. *T. lewisi* was obtained from batches of two or three rats when the trypanosomes were in either the reproductive or the adult phase. Rats were bled 3–5 days after inoculation for the reproductive phase and 7–9 days after inoculation for the adult phase. *T. equiperdum* was obtained from rats infected 2 or 3 days previously and bled when the trypanosomes were numerous in the blood. One rat provided ample material for eight flasks.

Infected rats were chloroformed and the blood taken immediately by heart puncture into a solution containing 0·85 % (w/v) of sodium chloride and 1·5 % (w/v) of sodium citrate. The trypanosomes were separated from the blood by differential centrifugation in a small angle centrifuge. The blood was centrifuged at 1800 r.p.m. for 3 min. and the white supernatant containing the trypanosomes was pipetted off from the red blood cells. The suspension was then centrifuged at 2800 r.p.m. for 2 min. and the trypanosomes were resuspended in citrate saline solution. With *T. equiperdum*, glucose was added at this first washing to give an 0·002 M solution; it was not added to *T. lewisi*. Ryley (1956) also found it necessary to wash *T. equiperdum* with a solution containing glucose. The trypanosomes were washed once more with citrate saline solution, once with modified Ringer–phosphate solution and were finally suspended in modified Ringer–phosphate solution. After each centrifugation, the small deposit of red blood cells was left behind and the final suspension contained fewer than one red blood cell to every 100 trypanosomes.

The modified Ringer–phosphate solution used for *T. lewisi* was prepared by mixing 21 ml. of salt solution of the following composition: 0·146 M-NaCl, 0·0059 M-KCl, and 0·00146 M-$MgSO_4 \cdot 7H_2O$, with 23 ml. of 0·1 M phosphate buffer pH 7·5–7·6 prepared from Na_2HPO_4 and KH_2PO_4. The oxygen uptake of trypanosomes in this solution was slightly higher than in solutions at pH 7·0–7·2. The proportion of buffer to salt solution was not critical; the above proportions were the most convenient to prepare from concentrated solutions. This solution had insufficient buffering power for *T. equiperdum* and the increase in acidity to pH 6·8 or less at the end of the first hour of experiments adversely affected the O_2 consumption. For *T. equiperdum*, therefore, the phosphate content of the solution was increased by mixing equal volumes of 0·1 M phosphate buffer, pH 7·6, and the above modified Ringer–phosphate solution. With this buffer the pH dropped to only 7·4 after $2\frac{1}{2}$ hr. when there were fewer than $3{\cdot}0 \times 10^7$ trypanosomes per flask, but dropped to 7·2 after $2\frac{1}{2}$ hr. when there were $5{\cdot}0 \times 10^7$ trypanosomes per flask.

The oxygen consumption was measured with differential manometers using microflasks of 5 ml. capacity. The number of trypanosomes in the suspension was

measured in an absorptiometer before the flasks were set up, and the concentration adjusted so that each flask contained 2–5 × 10^7 trypanosomes. A further sample of the suspension was diluted again and counted in a haemocytometer. The total volume of reaction mixture in the main compartment of each flask was 0·9 ml., and each flask also contained 0·1 ml. 15% KOH in the centre well, absorbed on a roll of filter-paper. Glucose and other substrates were dissolved in the same Ringer–phosphate solution as that in which the trypanosomes were suspended. The flasks were equilibrated in air at 37° C. for 15 min.; the taps were then closed and manometric readings commenced. The time taken between bleeding the rats and pipetting the suspension of washed trypanosomes into the flasks was usually about 1 hr.

The trypanosomes were examined microscopically at the end of each experiment.

Measurements of pH were made with a Pye Universal pH meter and a micro-glass electrode designed by Hartree (1952).

Ammonia was estimated by the colorimetric method of Johnson (1941), described by Umbreit, Burris & Stauffer (1949). Because the trypanosomes were suspended in Ringer–phosphate solution, aliquots of experimental solution and standard ammonium chloride were diluted with Ringer–phosphate solution and water to give a total volume of 1 ml. Ringer–phosphate solution and 1 ml. water, instead of 2 ml. water. To this was added 2 ml. colour reagent, containing KI, HgI_2 and gum ghatti, and then 3 ml. 2 N-NaOH was added.

III. OXYGEN UPTAKE IN THE ABSENCE OF SUBSTRATE

(1) *Trypanosoma lewisi*

The washed trypanosomes were still motile when the suspension was pipetted into the flasks. In the total absence of substrate, motility decreased slowly, the trypanosomes becoming non-motile in about 60 min. Oxygen consumption decreased at the same time as motility. Thus, from 0 to 30 min. the rate of O_2 consumption for adult phase *T. lewisi* was 18–23 μl./10^8 trypanosomes/hr.; from 30 to 60 min., 9–13 μl./hr., and from 60 to 90 min., 7–12 μl./hr., giving a rate of 9–15 μl./10^8/hr. over the first 2 hr. This compares with the previously reported figure of 4·0 μl./10^8/hr. over the first 2 hr. (Ryley, 1951) and the observation that O_2 consumption almost ceased after the first 15 min. (Moulder, 1948*a*).

If glucose were tipped into the reaction mixture after 30 min., when the O_2 consumption of the trypanosomes was very low, the rate of O_2 consumption increased rapidly, and after 10 min. reached the same rate as in flasks where glucose had been present since the beginning of the experiment, as is shown in Fig. 1. The concentration of glucose in both flasks was 0·02 M.

Moulder (1948*a*) found that a suspension of *T. lewisi* could be stored without glucose at 0° for 30 min. without damaging the trypanosomes. In the present experiments, suspensions of *T. lewisi* in Ringer–phosphate solution were left at 5° C. for 24 hr. without glucose. The oxygen uptake of these trypanosomes was then measured at 37° C., with and without added glucose, and compared with the previously determined oxygen uptake of aliquots from the same suspension when freshly prepared. When no glucose was added, the oxygen consumption was about

half of that of the previous day. When glucose was added, the curve of the previous day, with glucose, was almost duplicated, showing that the trypanosomes suffered little damage when stored without glucose at this low temperature.

(2) *Trypanosoma equiperdum*

Trypanosomes of the *evansi-brucei* group are dependent on glucose or other utilizable substrate for motility. Ryley (1956) says that 'loss of motility on the removal of substrate coincides with a loss of respiratory activity, and is soon followed by cellular disintegration'. Williamson & Rollo (1952) found that *T. rhodesiense* survived only 30 min. in a Ringer–phosphate solution.

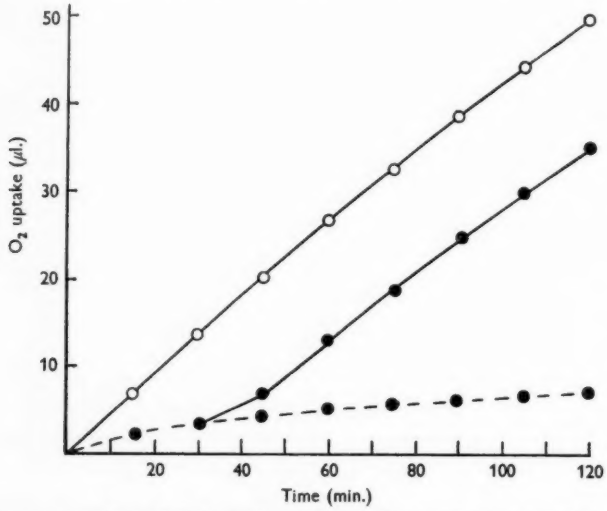

Fig. 1. Oxygen consumption of *T. lewisi* in modified Ringer–phosphate solution. The broken line represents O_2 uptake in the absence of substrate. The solid lines represent O_2 uptake with 0·02 M glucose. In ○—○, glucose was present from the beginning of the experiment. In ●—●, glucose was tipped into the reaction mixture after 30 min. Each flask contained $3{\cdot}7 \times 10^7$ trypanosomes; 6th-day infection.

In the present investigation it was found that trypanosomes washed once with citrate saline solution containing 0·002 M glucose and twice with solutions lacking a substrate still showed slight motility when introduced into the flasks. In the absence of substrate, the trypanosomes became non-motile within 10 min. and no O_2 consumption was observed after the 15 min. equilibration period. Cellular disintegration took place within a further 30–45 min.

Rapid motility was quickly regained if the suspension of trypanosomes showing slight motility were introduced into flasks containing a substrate. Unlike *T. lewisi*, no motility was regained and no O_2 consumption was observed if glucose were tipped into the reaction mixture immediately after the 15 min. equilibration period. Harvey (1949) similarly found that increased O_2 consumption was only occasionally observed if the substrate were tipped after a 5 min. equilibration

period. Harvey worked with *T. hippicum*, which is probably a strain of *T. evansi* (Hoare & Bennett, 1937).

Unlike *T. lewisi*, *T. equiperdum* did not regain motility if the suspension were left at 5° C., for 15 min. before substrate was added.

IV. EFFECT OF SINGLE SUBSTRATES ON OXYGEN UPTAKE

Substrates were present in the flasks from the beginning of the experiments and the concentration of substrate in the reaction mixture was 0·02 M, unless otherwise stated.

(1) *Trypanosoma lewisi*

(*a*) *Comparison of adult and reproductive phases.* Moulder (1948*b*) showed that, with glucose as substrate, suspensions of washed *T. lewisi* had a more rapid rate of O_2 consumption in the adult phase than in the reproductive phase, consuming 75 μl. $O_2/10^8$ trypanosomes/hr. at 7–8 days after inoculation, but only 47 μl. at 3–4 days. This was confirmed by Zwisler & Lysenko (1954) with figures of 75·7 μl. $O_2/10^8$/hr. at 7 days and 62·7 μl. at 4 days.

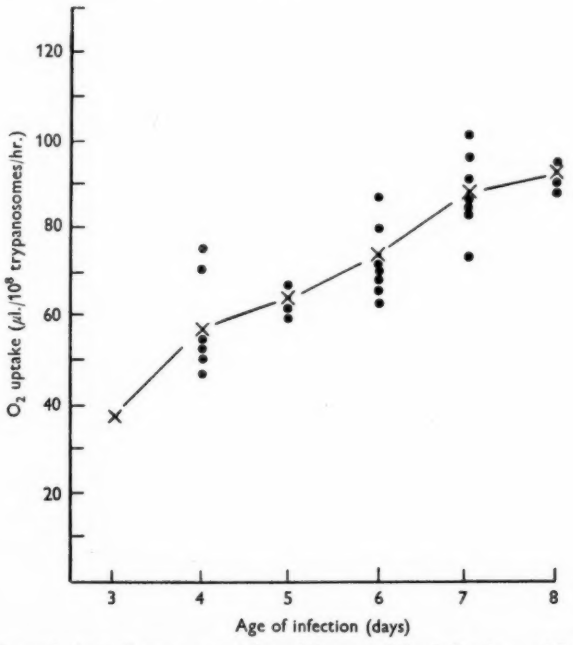

Fig. 2. *T. lewisi*: variation of oxygen uptake with age of the infection, with glucose as substrate. Each experiment is shown by a solid circle. The mean oxygen uptake for each day is shown by a cross.

The present investigation again confirmed this difference between adult and reproductive phase trypanosomes. Fig. 2 shows the oxygen consumption of trypanosomes taken from rats 3–8 days after inoculation, with glucose as substrate.

The mean rate at 7 days was 88 μl./10^8/hr.; at 5 days it was 63·5 μl., and at 4 days it was 56·1 μl. The rate of consumption of oxygen decreased slowly but continuously throughout each experiment, as is shown in Fig. 1. The trypanosomes were still very active after 2 hr., and the O_2 uptake in the second hour of observation was about 85 % of that of the first hour: Moulder (1948*a*) observed that it was 89 % of that of the first hour. Zwisler & Lysenko (1954) found a slight decrease in O_2 uptake during the first hour.

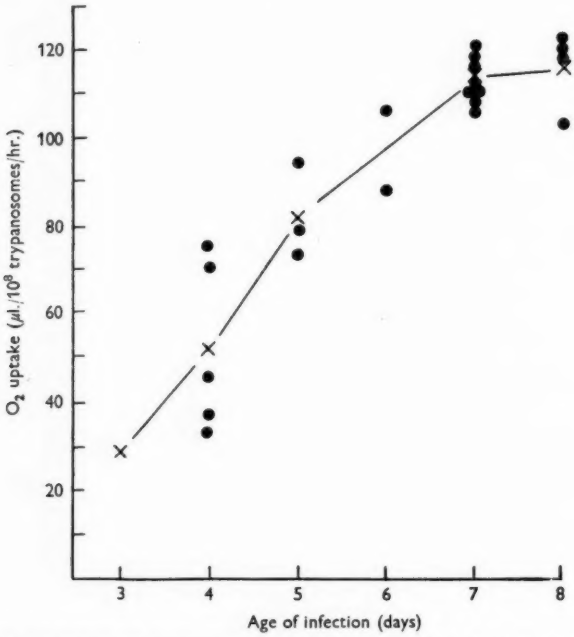

Fig. 3. *T. lewisi*: variation of oxygen uptake with age of the infection, with glutamine as substrate. Each experiment is shown by a solid circle. The mean oxygen uptake for each day is shown by a cross.

In the same way, the variation of oxygen uptake with age of the infection was investigated with glutamine as substrate instead of glucose; the results are shown in Fig. 3. With glutamine, the mean rate at 7 days was 113 μl. O_2/10^8/hr., and at 4 days was only 52·6 μl. That is, at 7 days the oxygen uptake with glutamine was 128 % of that with glucose, whereas at 4 days the oxygen uptake with glutamine was only 93·8 % of that with glucose. In addition, adult phase trypanosomes with glutamine as substrate were still very active after 2 hr., whereas reproductive phase trypanosomes with glutamine were definitely less active than those with glucose.

Table 1 shows the results of five experiments with 4th-day trypanosomes and five experiments with 7th-day trypanosomes, in which the oxygen uptake with glucose was compared with the oxygen uptake with glutamine as substrate. These results have been included in Figs. 2 and 3. The O_2 uptake of 7th-day trypanosomes

Table 1. *The results of five experiments with trypanosomes at the 4th day of infection and five experiments with trypanosomes at the 7th day of infection, in which the oxygen uptake with glucose and glutamine as substrates was compared. These results have been included in Figs.* 2 *and* 3

	Glucose			Glutamine			O_2 uptake with glutamine as percentage of O_2 uptake with glucose
Day of infection	Concentration (M)	O_2 uptake (μl./10^8/hr.)	Motility at 2 hr.	Concentration (M)	O_2 uptake (μl./10^8/hr.)	Motility at 2 hr.	
4th	0·02	52·8	Very active	0·005	46·4	Active	87·8
		50·0	Very active	0·002	37·5	Active	75·2
		47·0	Very active	0·002	33·0	Active	70·3
		76·4	Very active	0·002	75·7	Active	99·2
		54·2	Very active	0·005	70·7	Very active	130·0
7th	0·02	85·3	Very active	0·005	117·5	Very active	137·8
		86·7	Very active	0·005	110·0	Very active	127·0
		85·0	Very active	0·002	110·0	Very active	129·3
		91·4	Very active	0·002	116·7	Very active	127·6
		88·8	Very active	0·002	106·0	Very active	119·3

with glutamine as substrate was 119–138% of the O_2 uptake with glucose as substrate. With 4th-day trypanosomes, three experiments showed that O_2 uptake with glutamine was considerably less than with glucose; in one experiment both glucose and glutamine gave higher O_2 uptakes than usual, but motility after 2 hr. was less with glutamine than with glucose; in one other experiment, the O_2 uptake with glutamine was appreciably higher than with glucose and more characteristic of the adult phase trypanosomes.

Moulder (1948*a*) obtained different results when comparing glucose and glutamine metabolism. He found that at 5–7 days the oxygen uptake with glutamine was 123% of that with glucose, but that at 4 days the oxygen uptake with glutamine was 148% of that with glucose. Ryley (1951), working with 10th-day *T. lewisi*, found that the oxygen uptake with glutamine was 121·5% of that with glucose.

(*b*) *Oxygen uptake with amino-acids and other compounds.* It has been shown above that the average oxygen consumption with glutamine as substrate was 113 μl./10^8/hr. for a 7-day infection and 52·6 μl. for a 4-day infection. Moulder (1948*a*) gave 88·3 and 73 μl. respectively, while Ryley (1951) gave 72·9 μl. for a 10-day infection, compared with 60·0 μl. with glucose as substrate.

In the present investigations it was found that 0·002 and 0·01 M solutions of glutamine gave the same rate of oxygen uptake over 2 hr. (with both adult and reproductive phase trypanosomes) provided there were fewer than 5×10^7 trypanosomes per flask. Most of the experiments shown in Fig. 3 were performed with 0·002 M glutamine.

Other amino-acids and related substances were tested for their effect on the O_2 consumption of adult phase *T. lewisi*. Glucosamine, 0·02 M, gave an O_2 uptake of about 63 μl./10^8 trypanosomes/hr. Slight O_2 uptake was observed with 0·02 M-Na L-glutamic acid, 0·01 M asparagine, 0·01 M aspartic acid, 0·2% (w/v) Difco Bacto-

peptone, 0·2% (w/v) casein hydrolysate and 0·2% (w/v) yeast extract. With all these substances the O_2 uptake for the first hour of experiment was between 18 and 30 μl./10^8/hr., and the trypanosomes were slightly more active than in the absence of substrate.

The following compounds did not support motility when tested at a final concentration of 0·2% (w/v): L-arginine HCl, L-cystine, glycine, L-histidine HCl, L-leucine, DL-*iso*lecuine, L-lysine HCl, DL-methionine, L-phenylalanine, DL-serine, DL-tryptophane, DL-tyrosine, and L-valine. These tests on motility were carried out with suspensions of washed trypanosomes in small corked tubes that were incubated at 37° C., and not in manometer flasks.

Ryley (1951) found that asparagine and glutamic acid were oxidized slowly but that alanine, aspartic acid and glycine did not increase the uptake of oxygen. Moulder (1948*a*) found that DL-alanine was not oxidized, while L-aspartate and L-asparagine were oxidized slowly and L-glutamate gave as high an O_2 uptake as did glucose.

(2) *Trypanosoma equiperdum*

(*a*) *Oxygen uptake with glucose and glycerol.* It is well known that trypanosomes of the *evansi-brucei* group show a high rate of oxygen consumption when glucose is present. Reiner, Smythe & Pedlow (1936) pointed out that when suspensions of washed *T. equiperdum* metabolized glucose, the oxygen uptake decreased continuously and absolute figures were therefore difficult to obtain. They recorded an O_2 uptake of 235 μl./10^8 trypanosomes/hr. for the first 15 min.; but only 91 μl. for the period 15–45 min. Harvey (1949) likewise obtained an initial O_2 uptake of 150–250 μl., and found that the O_2 consumption after 1 hr. was less than half of the original rate.

In the present experiments, the average oxygen uptake over the first hour was 138 μl./10^8/hr. (average of ten experiments with extreme values 127 and 160 μl.), given uniform conditions such as buffer, numbers of trypanosomes, concentration of glucose (0·02 M) and time taken washing the trypanosomes. Occasionally, blood was taken from rats or mice with exceptionally heavy infections. In these cases the oxygen uptake was lower, being between 70 and 100 μl./10^8/hr. over the first hour.

The oxygen uptake decreased continuously and rapidly as is shown in the upper curve of Fig. 4. The average rate for the first 15 min. was 169 μl./10^8/hr. (150–195 μl.), which was only slightly less than in the presence of serum. The rate for the second hour varied considerably. In three experiments it was 52, 45 and 82 μl., compared with O_2 uptakes of 138, 128 and 160 μl. for the first hour. Decrease in O_2 uptake during the second hour was most marked in the flasks with the greater number of trypanosomes.

Oxygen uptake with glycerol was slightly lower than with glucose, being 120 μl./10^8/hr. over the first hour (average of ten experiments, extreme values 92 and 152 μl.). Oxygen uptake with a mixture of 0·02 M glucose plus 0·02 M glycerol was at the same rate as with glucose alone. The effect of glycerol was first recorded by Kudicke & Evers (1924) in a study of motility. Marshall (1948) and Harvey (1949) found that it increased oxygen uptake to the same extent as glucose.

It has been shown above that no oxygen uptake was observed when *T. equiperdum* was maintained at 5° in the absence of substrate. If, however, the trypanosomes were suspended in Ringer–phosphate solution containing glucose or glycerol, considerable oxygen uptake was observed when the trypanosomes were tested at 37° C. after being stored for 22–24 hr. at 5° C. Fig. 4 shows the results of one such experiment. The broken lines represent the oxygen uptake of $3{\cdot}0 \times 10^7$ washed

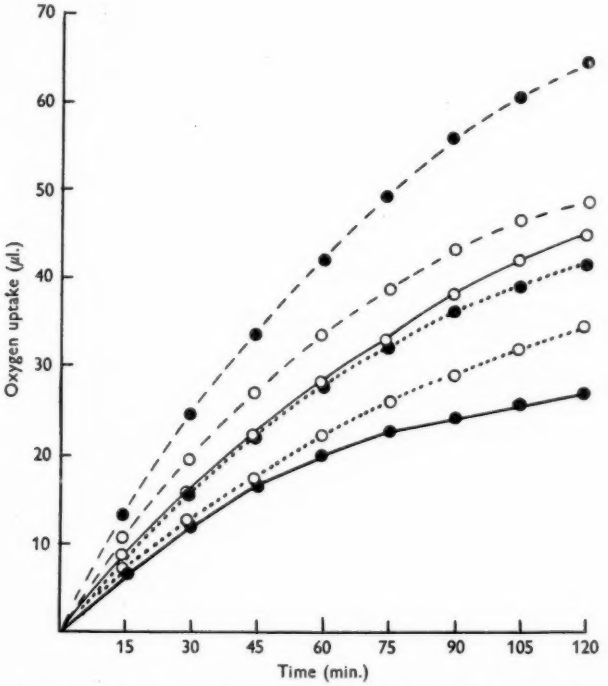

Fig. 4. Oxygen uptake of *T. equiperdum* with 0·02 M glucose or 0·02 M glycerol in Ringer–phosphate solution at 37° C. Each flask contained $3{\cdot}0 \times 10^7$ trypanosomes. ●, Glucose; ○, glycerol. Broken lines indicate O_2 uptake of freshly prepared suspension. Dotted lines indicate O_2 uptake of same suspension after 22½ hr. at 5° C. Unbroken lines indicate O_2 uptake of suspension prepared from citrated infected blood after 24½ hr. at 5° C.

trypanosomes with 0·02 M glucose or 0·02 M glycerol as substrate, using a freshly prepared suspension and equilibrating for 14 min. Glucose gave an oxygen uptake of 140 μl. $O_2/10^8$ trypanosomes during the first hour, and glycerol gave 111 μl.

Similar aliquots of the same suspension were pipetted at the same time into chilled flasks containing glucose or glycerol and these were placed at 5° C. for 22½ hr. The oxygen uptake of the trypanosomes in these flasks, at 37° C. after a 14 min. equilibration period, is shown by the dotted curves. Both with glucose and glycerol the oxygen uptake was considerably less than that of the original suspension; glucose gave 91 μl./10^8/hr. and glycerol gave 73 μl.

When the infected blood was first taken from the heart of the rat, some was

pipetted into citrate saline containing glucose and placed immediately at 5° C. After 24½ hr. a suspension of washed trypanosomes was prepared from this citrated blood by differential centrifugation in the usual way. The oxygen uptake with glucose or glycerol was again measured at 37° C. after 14 min. equilibration. During the first hour, glucose gave an O_2 uptake of 67 μl./10^8 trypanosomes and glycerol gave 94 μl.; that is, the oxygen uptake with glucose was much reduced but the oxygen uptake with glycerol approached that of the original suspension. There were $3{\cdot}7 \times 10^7$ trypanosomes per flask. The figures were recalculated on a basis of $3{\cdot}0 \times 10^7$ trypanosomes per flask and are shown in Fig. 4 by the unbroken lines.

(*b*) *Oxygen uptake with amino-acids and other compounds.* Very slight oxygen uptake was recorded with 0·2% (w/v) Difco yeast extract, 0·2% (w/v) casein hydrolysate and 0·2% (w/v) Difco Bacto-peptone. No oxygen uptake was recorded with glutamine, Na L-glutamic acid, 0·01 M asparagine, 0·01 M aspartic acid, DL-alanine, 0·02 M and 0·002 M-Na acetate, or glucosamine. These substances were then tested for their effect on the motility of *Trypanosoma equiperdum*. Suspensions of washed trypanosomes were prepared in the usual way, but were set up in small corked tubes instead of in manometer flasks. The tubes were incubated at 37° C. and examined at 10 or 15 min. intervals. No motility was observed in any of the tubes except those with yeast extract, casein hydrolysate or Bacto-peptone as substrate. With yeast extract and casein hydrolysate the activity after 15 min. was almost as intense as with glucose; with Bacto-peptone, only moderate activity was observed. After 30 min. the trypanosomes were all only slightly motile and they were non-motile after 45 min. The initial high activity was not observed during manometric experiments because about 30 min. elapses between the introduction of the trypanosomes into the flasks with the substrate and the first readings of the manometers.

The following compounds were tested for their effect on the motility of *T. equiperdum* but were not tested for their effect on O_2 consumption in manometers: L-arginine HCl, L-cystine, glycine, L-histidine HCl, L-leucine, DL-*iso*leucine, L-lysine HCl, DL-methionine, L-phenylalanine, DL-serine, DL-tryptophane, DL-tyrosine and L-valine. These compounds were tested at a concentration of 0·2% (w/v) and no motility was observed in any tube. The trypanosomes were, however, motile if glucose was also present. This showed that the amino-acids were not toxic at that concentration.

Marshall (1948) found that *T. evansi* did not oxidize glutamate or aspartate. Earlier work on the motility of trypanosomes (Kudicke & Evers, 1924) or the utilization of substrates (von Brand, 1933) had not included amino-acids, and the experiments of Manozzi-Torini (1940) showed the effect of amino-acids on the oxygen consumption of *T. evansi* with glucose as substrate, and not the oxygen uptake with amino-acids in the absence of glucose.

Similarly, the experiments of Williamson & Rollo (1952) on *T. rhodesiense* showed the effect of amino-acids on motility in the presence of glucose, except that 0·2% Difco Bacto-peptone in buffered Ringer solution was found to support motility for 5 hr. either with or without glucose. The concentration of trypanosomes used in their work was probably appreciably less than in the present experiments.

The present experiments show that amino-acids which cause an increase in oxygen uptake when tested with *T. lewisi* have no effect on the oxygen uptake of *T. equiperdum*.

V. THE EVOLUTION OF AMMONIA DURING METABOLISM OF GLUTAMINE

(1) *Liberation of ammonia from glutamine*

Solutions of glutamine were first tested to see whether they gave any colour reaction in the absence of trypanosomes. Very little colour was observed immediately after the sodium hydroxide solution had been added to the reaction mixture. Ammonia was, however, liberated from glutamine in this alkaline solution and the colour

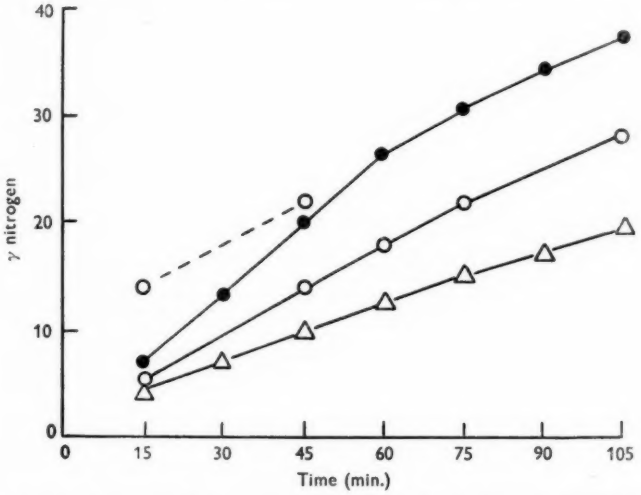

Fig. 5. Liberation of ammonia from glutamine during colorimetric estimation. Sodium hydroxide was added at 0 min. △—△, 0·32 mg. glutamine; ○—○, 0·42 mg., ●—●, 0·64 mg. The broken line represents 0·42 mg. glutamine when maintained at 50–60°.

became more intense. Solutions containing between 0·29 and 0·64 mg. glutamine were tested for ammonia at 15 min. intervals for up to 105 min. The rate of evolution of ammonia is shown in Fig. 5. In thirteen estimations, 15–20·4 μg. N was liberated per mg. glutamine between 30 and 60 min. after the addition of sodium hydroxide; six of these estimations gave 16·5–17·6 μg. N. Similar results were obtained when the glutamine was incubated at 37° for 2 hr. in Ringer–phosphate solution at pH 7·6 or 8·7 before being tested for ammonia.

When the reaction mixture containing glutamine was maintained at 50–60° after the addition of sodium hydroxide, the rate of liberation of ammonia was greatly accelerated during the first 15 min. but then continued at the usual rate, as is shown by the broken line in Fig. 5. Raising the temperature to 100° C. reduced the colour reaction and caused the solution to become cloudy.

Solutions containing 0·6 or 3·0 mg. L-glutamic acid were also tested. They gave no colour reaction at room temperature or when heated to 50–60°.

(2) *Liberation of ammonia by* Trypanosoma lewisi

Ryley (1951) stated that *Trypanosoma lewisi* liberates free ammonia during the oxidation of glutamine. Fig. 6 shows the O_2 uptake of 8th-day *T. lewisi* with glutamine as substrate: each flask contained $3{\cdot}0 \times 10^7$ trypanosomes. The trypanosomes were suspended in Ringer–phosphate solution and equilibrated at 37° C. for 15 min. The taps were then closed, the first manometric readings recorded, and

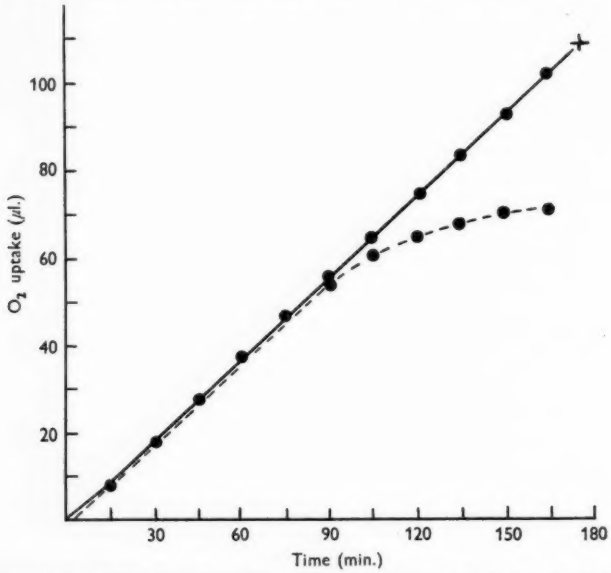

Fig. 6. Oxygen uptake of 8th-day *T. lewisi* with glutamine as substrate. Each flask contained $3{\cdot}0 \times 10^7$ trypanosomes. Flasks shown by the broken line contained 0·127 mg. glutamine. Flasks shown by the unbroken line contained 1·27 mg. glutamine.

glutamine was tipped from the side-bulb at 0 min. The broken line indicates the mean of three flasks into which 0·127 mg. glutamine was tipped; the solid line indicates the mean of three flasks into which 1·27 mg. glutamine was tipped. Oxygen uptake commenced at the same rate in the two series, but decreased after about 90 min. in the flasks with only 0·127 mg. glutamine, when about 54 μl. O_2 had been consumed. At 175 min. the contents of the flasks were centrifuged for 5 min. and the clear supernatant used for the estimation of ammonia.

Control suspensions of trypanosomes in Ringer–phosphate solution were kept at 37° C. or 5° C. for 2 hr.; they were also centrifuged and tested for ammonia. Negligible readings were recorded.

Estimations of ammonia were started 15 min. after the addition of sodium hydroxide and continued at intervals for 90 min. Fig. 7 shows the results with 0·3 ml. aliquots from the flasks which originally contained 1·27 mg. glutamine in 0·9 ml., and 0·6 ml. aliquots from flasks which originally contained 0·127 mg. glutamine in 0·9 ml.: the control estimation of 0·42 mg. glutamine is also included. Extrapolation of the experimental curves in Fig. 7 back to 0 min. gives 21·75 μg. N

liberated from 0·127 mg. glutamine by *T. lewisi* in 175 min., and 50 μg. N liberated from 1·27 mg. glutamine.

By extrapolating the curves in Fig. 6 to 175 min., it is seen that 50 μg. N was liberated during the consumption of 108 μl. O_2. At the same rate, 47 μl. O_2 would be consumed during the liberation of 21·75 μg. N; that is, utilization of the lower concentration of glutamine continued for about 80 min. and then was followed by a decreased oxygen uptake in the absence of substrate. If the trypanosomes had utilized all the glutamine, then 21·75 μg. N would have been liberated from 127 μg. glutamine. The theoretical yield of 2 atoms of nitrogen from each molecule of glutamine is 24·3 μg. from 127 μg. glutamine.

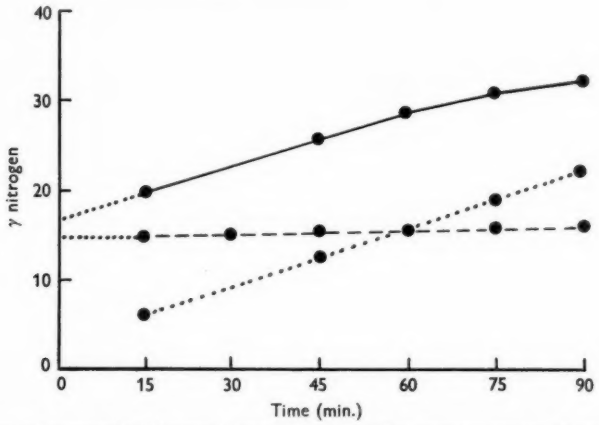

Fig. 7. Estimation of ammonia liberated from glutamine by trypanosomes shown in Fig. 6. Sodium hydroxide was added at 0 min. The broken line shows the liberation of ammonia from 0·6 ml. aliquots from the flasks that originally contained 0·127 mg. glutamine in 0·9 ml. The unbroken line shows the liberation of ammonia from 0·3 ml. aliquots from the flasks that originally contained 1·27 mg. glutamine in 0·9 ml. The dotted line shows the control estimation of 0·42 mg. glutamine.

A considerable amount of glutamine remained in the flasks that had originally contained 1·27 mg. glutamine, and ammonia was liberated from this glutamine while experiments for determining the amount of ammonia liberated by the trypanosomes were being performed. By comparing the slope of this curve with that of control solutions of glutamine, it was possible to estimate the approximate amount of glutamine remaining in solution, and therefore the amount consumed by the trypanosomes. In the experiments shown in Figs. 6 and 7, $3{\cdot}0 \times 10^7$ trypanosomes consumed approximately 290 μg. glutamine, with a corresponding uptake of 108 μl. O_2 and liberation of 50 μg. N in the form of ammonia. The theoretical yield of N from 290 μg. glutamine would be 55·5 μg.

These experiments were repeated twice and gave similar results.

The experiments were also repeated with 4th- and 5th-day *T. lewisi*, with 1·27 mg. glutamine per flask.

$2{\cdot}0 \times 10^7$ 4th-day trypanosomes liberated 22·5 μg. N in 220 min., with a corresponding uptake of 36 μl. O_2.

$1{\cdot}8 \times 10^7$ 5th-day trypanosomes liberated 33 μg. N in 350 min., with a corresponding uptake of 57·5 μl. O_2.

$1{\cdot}8 \times 10^7$ 5th-day trypanosomes liberated 25·5 μg. N in 270 min., with a corresponding uptake of 45 μl. O_2.

Calculating the above figures in terms of 100 μg. N, 4th-day trypanosomes consumed 160 μl. O_2/100 μg. N, 5th-day trypanosomes consumed 174 and 176 μl., and 8th-day trypanosomes consumed 216 μl. Adult phase trypanosomes therefore consumed more oxygen than reproductive phase trypanosomes during the liberation of equal amounts of ammonia.

VI. DISCUSSION

Trypanosoma lewisi and *T. equiperdum* were found to differ in many respects, some of which affected the planning of experiments. First, it was necessary to use a more highly buffered Ringer–phosphate solution when working with *T. equiperdum* because of the greater production of acids as metabolic end-products.

Secondly, no oxygen uptake was recorded with *T. equiperdum* in the absence of substrate, and the power to regain respiration on the addition of substrate was rapidly lost, so that it was essential for substrate to be present in the flasks from the beginning of the experiments. With *T. lewisi*, however, oxygen uptake continued at a low but decreasing rate for up to 2 hr. in the absence of substrate, and increased oxygen uptake was observed if substrate were tipped into the reaction mixture 20 or 30 min. after manometric readings had begun. At this time the endogenous rate of oxygen uptake was at a very low level and the effect of substrates which increased oxygen uptake only slightly or for brief periods was more readily observed than if they had been present in the reaction mixture since the beginning of the experiment.

The differences in the absence of substrate were also seen in the effect of low temperatures. Suspensions of *T. lewisi* survived for 24 hr. at 5° C. without substrate, whereas suspension of *T. equiperdum* did not. *T. equiperdum* did, however, survive for 24 hr. at 5° C. if glucose or glycerol were present, but the rate of oxygen uptake was considerably lower than the original rate.

There was a fundamental difference in the effect of amino-acids and related substances. With *T. lewisi*, glutamine and glucosamine promoted a high rate of oxygen uptake, and several other substances also caused an increase in oxygen uptake, but at a slower rate, while with *T. equiperdum* oxygen uptake was observed with only yeast extract, Difco Bacto-peptone and casein hydrolysate.

VII. SUMMARY

1. Standard conditions are described for preparing suspensions of washed *Trypanosoma lewisi* and *T. equiperdum* in modified Ringer–phosphate solution.

2. Oxygen consumption was measured with differential manometers, using microflasks containing $2\text{–}5 \times 10^7$ trypanosomes in 0·9 ml. of reaction mixture. Measurements of oxygen uptake were carried out at 37° C.

3. *T. lewisi* respired slowly in the absence of substrate for up to 2 hr. The try-

panosomes suffered little damage when stored at 5° C. for 24 hr. without substrate. No oxygen uptake was observed with *T. equiperdum* in the absence of substrate. The trypanosomes were viable after 24 hr. at 5° C. with glucose or glycerol as substrate, but not in the absence of substrate.

4. With glucose as substrate, the rate of oxygen consumption by *T. lewisi* increased with the age of infection. This change was more marked with glutamine as substrate.

5. With glucosamine as substrate, the oxygen uptake of *T. lewisi* was at a slightly lower rate than with glucose. The rate of oxygen uptake was still lower with Na L-glutamic acid, asparagine, aspartic acid, casein hydrolysate, yeast extract and Difco Bacto-peptone. Thirteen other amino-acids had no effect on the motility of the trypanosomes.

6. With glycerol as substrate, the oxygen uptake of *T. equiperdum* was at a slightly lower rate than with glucose. The rate of oxygen uptake was very low with yeast extract, casein hydrolysate and Difco Bacto-peptone. No oxygen uptake or motility was recorded with glutamine, Na L-glutamic acid, glucosamine, asparagine, aspartic acid, DL-alanine, or Na acetate. Thirteen other amino-acids had no effect on the motility of the trypanosomes.

7. Ammonia was liberated from glutamine by adult and reproductive phase *T. lewisi*.

I should like to thank Dr P. Tate for helpful advice during the course of this work, and Dr H. Laser for teaching me manometric methods. This work was commenced while I held the Tucker-Price Research Fellowship of Girton College, Cambridge, and was continued with a grant from the Agricultural Research Council.

REFERENCES

Brand, T. von (1933). Studien über den Kohlenhydratstoffwechsel parasitischer Protozoen. II. Der Zuckerstoffwechsel der Trypanosomen. *Z. vergl. Physiol.* **19**, 587.

Brand, T. von (1951). *Biochemistry and Physiology of Protozoa*, vol. I, p. 177. Ed. by Lwoff, A. New York: Academic Press.

Hartree, E. F. (1952). A micro glass electrode. *Biochem. J.* **52**, 619.

Harvey, S. C. (1949). The carbohydrate metabolism of *Trypanosoma hippicum*. *J. biol. Chem.* **179**, 435.

Hoare, C. A. & Bennett, S. C. J. (1937). Morphological and taxonomic studies on mammalian trypanosomes. III. Spontaneous occurrence of strains of *Trypanosoma evansi* devoid of the kinetonucleus. *Parasitology*, **29**, 43.

Johnson, M. J. (1941). Isolation and properties of a pure yeast polypeptidase. *J. biol. Chem.* **137**, 575–86.

Kudicke, R. & Evers, E. (1924). Über den Einfluss von Zuckerarten und Alkoholen der Zuckerreihe auf die Beweglichkeit der Trypanosomen *in vitro*. *Z. Hyg. InfektKr.* **101**, 317.

Mannozzi-Torini, M. (1940). Alcune osservazioni sopra il metabolismo del *Trypanosoma evansi*. *Arch. Sci. biol.* (*Napoli*), **26**, 565.

Marshall, P. B. (1948). The glucose metabolism of *Trypanosoma evansi* and the action of trypanocides. *Brit. J. Pharmacol.* **3**, 8.

Moulder, J. W. (1948*a*). The oxidative metabolism of *Trypanosoma lewisi* in a phosphate-saline medium. *J. infect. Dis.* **83**, 33.

Moulder, J. W. (1948*b*). Changes in the glucose metabolism of *Trypanosoma lewisi* during the course of infection in the rat. *J. infect. Dis.* **83**, 42.

REINER, L., SMYTHE, C. V. & PEDLOW, J. T. (1936). On the glucose metabolism of trypanosomes (*Trypanosoma equiperdum* and *Trypanosoma lewisi*). *J. biol. Chem.* **113**, 75.

RYLEY, J. F. (1951). Studies on the metabolism of the Protozoa. 1. Metabolism of the parasitic flagellate, *Trypanosoma lewisi*. *Biochem. J.* **49**, 577.

RYLEY, J. F. (1956). Studies on the metabolism of the Protozoa. 7. Comparative carbohydrate metabolism of eleven species of trypanosome. *Biochem. J.* **62**, 215.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, p. 161. Minneapolis: Burgess Publishing Co.

WILLIAMSON, J. & ROLLO, I. M. (1952). Stimulating effect of amino-acids on the survival at 37° C. of *Trypanosoma rhodesiense* in a serum-free synthetic medium. *Nature, Lond.*, **170**, 376.

ZWISLER, J. B. & LYSENKO, M. G. (1954). The oxidative metabolism of *Trypanosoma lewisi* from salicylate treated infected white rats. *J. Parasit.* **40**, 531.

(*MS. received for publication* 24. v. 1957. Ed.)

THE EFFECT OF SOME METABOLIC INHIBITORS ON THE OXYGEN UPTAKE OF *TRYPANOSOMA LEWISI* AND *TRYPANOSOMA EQUIPERDUM*

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(With 14 Figures in the Text)

CONTENTS

		PAGE
I.	Introduction	165
II.	Materials and methods	166
III.	The effect of mouse or rat blood or serum on the oxygen uptake of trypanosomes	166
	(1) The oxygen uptake of trypanosomes in heparinized infected blood	166
	(2) The effect of mouse serum on the oxygen uptake of washed trypanosomes	167
	(3) The effect of mouse blood on the oxygen uptake of washed trypanosomes	169
IV.	The effect of iodoacetic acid on oxygen uptake	170
	(1) The effect of iodoacetic acid on normal rat blood	170
	(2) The effect of iodoacetic acid on the oxygen uptake of *Trypanosoma lewisi*	173
	(3) The effect of iodoacetic acid on the oxygen uptake of *Trypanosoma equiperdum*	175
V.	The effect of arsenicals on the oxygen uptake of *Trypanosoma lewisi* and *T. equiperdum*	176
VI.	Discussion	181
VII.	Summary	181
	References	182

I. INTRODUCTION

Information about the nature of the metabolic processes of trypanosomes may be obtained by studying the action of inhibitors on the oxygen uptake of the parasites.

When sulphydryl inhibitors were tested on whole blood preparations, von Brand, Tobie & Mehlman (1950) found that trypanosomes of the *Trypanosoma evansi* and *T. brucei* groups were more sensitive than the *T. lewisi* group to all sulphydryl inhibitors tested. They concluded that enzymes containing sulphydryl groups probably play a more important role in the metabolism of the *T. evansi-brucei* group than the *T. lewisi* group.

Ryley (1956) examined the action of cyanide and iodoacetate on the oxygen uptake of six species of trypanosome. He compared the inhibition of respiration found in whole blood preparations with the inhibition in preparations of washed

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trypanosomes suspended in Ringer–phosphate–glucose solution; the degree of inhibition was similar in the two suspending media, apart from the case of *T. lewisi*. With *T. lewisi*, respiration was very little affected by iodoacetate in whole blood preparations, although it was inhibited strongly when the trypanosomes were suspended in inorganic media.

The purpose of the present investigation was to compare the effect of iodoacetic acid and arsenicals on the respiration of blood-stream forms of *T. lewisi* and *T. equiperdum* (*T. evansi-brucei* group) when the trypanosomes were suspended in solutions containing a variety of substrates.

II. MATERIALS AND METHODS

The strains of *T. lewisi* and *T. equiperdum* were the same as those used in previous experiments and suspensions of washed trypanosomes in modified Ringer–phosphate solutions were prepared in the way previously described (Thurston, 1957). *T. lewisi* was used 7–8 days after inoculation, when the trypanosomes were in the adult phase, except in one experiment when adult and reproductive phases of this trypanosome were compared.

When heparinized blood was required, 0·2% (w/v) heparin in 0·85% (w/v) sodium chloride solution was added at a rate of 0·25 ml./10 ml. blood. Mouse or rat serum was obtained by centrifuging the exudate from clotted blood. It was freshly prepared for each experiment.

The oxygen consumption of the trypanosomes was measured with differential manometers using microflasks of 5 ml. capacity. Each flask contained $2–5 \times 10^7$ trypanosomes. The total volume of reaction mixture in each flask was 0·9 ml. and each flask also contained 0·1 ml. 15% KOH in the centre well, absorbed on a roll of filter-paper. Glucose and other substrates or inhibitors were dissolved in the same modified Ringer-phosphate solution as that in which the trypanosomes were suspended. The flasks were equilibrated in air at 37° for 15 min.; the taps were then closed and manometric readings commenced. The trypanosomes were examined microscopically at the end of each experiment.

Solutions of arsenical compounds were prepared immediately before each experiment. The inhibitors, in 0·1 ml. Ringer–phosphate solution, were tipped into the reaction mixture 10 or 15 min. after manometric readings commenced. The preliminary non-inhibited oxygen uptake in these flasks was compared with that of the non-inhibited controls to ensure that the initial oxygen uptake was similar in all the flasks. After the inhibitor had been added, readings were continued for 1 hr. at 10 or 15 min. intervals.

III. THE EFFECT OF MOUSE OR RAT BLOOD OR SERUM ON THE OXYGEN UPTAKE OF TRYPANOSOMES

(1) *The oxygen uptake of trypanosomes in heparinized infected blood*

When experiments were carried out in the presence of whole blood, corrections for the oxygen uptake of the blood had to be applied, as was shown by von Brand & Tobie (1948).

In the present investigations, 0·6 ml. of heparinized normal rat blood was pipetted into the manometer flasks as soon as possible after the blood was withdrawn from the rat. Glucose, in Ringer–phosphate solution, was added to give 0·02M solution. The flasks were equilibrated at 37° for 15 min. The manometer stopcocks were closed and the first reading taken within 30 sec. of the closing of the stopcocks. An initial rapid rate of oxygen uptake was observed, as shown in Fig. 1, but after about 10 min. the oxygen uptake was reduced and continued at a constant rate for at least 2 hr.

The results of eight experiments with 0·6 ml. normal rat blood gave an average oxygen uptake of 12·6 μl. (extreme values 10·1 and 14·2 μl.) when read from 10–70 min. after manometric readings were commenced. When the initial rapid rate of oxygen uptake was included and readings taken from 0–60 min., the average uptake was 18·4 μl. This figure was more variable than the one for 10–70 min for the extreme values were 14·6 and 25·0 μl., and so the first 10 min. were excluded from most experiments where a correction was necessary. Michaelis & Salomon (1930), using washed rabbit erythrocytes, also found that the first stage of oxygen uptake sometimes showed irregularities.

The oxygen uptake of infected heparinized rat blood was determined in the manner described above for normal rat blood, and the above corrections applied for the 10–70 and 70–130 min. readings. The results for *T. lewisi* and *T. equiperdum* are shown in Table 1, where they are compared with the oxygen uptake of washed trypanosomes suspended in Ringer–phosphate–glucose solution. The oxygen uptake in each case was considerably higher in the heparinized blood than in the Ringer–phosphate solution. The difference between the oxygen uptake of reproductive phase (3 to 4-day) and adult phase (7 to 9-day) *T. lewisi* was previously shown with suspensions of the washed trypanosomes (Moulder, 1948*b*; Thurston, 1957). The present experiments show that it is also apparent in heparinized whole blood preparations.

The concentration of *T. equiperdum* affected the pH of the reaction mixture and the rate of oxygen uptake during the second hour of experiment. When there were $2–3 \times 10^7$ trypanosomes/flask, the pH at 130 min. was 7·3, and the oxygen uptake during the period 70–130 min. was 66% of that during the period 10–70 min. When there were $4–5 \times 10^7$ trypanosomes/flask, the pH at 130 min. was 7·0, and the oxygen uptake during the period 70–130 min. was only 48% of that during the period 10–70 min.

(2) *The effect of mouse serum on the oxygen uptake of washed trypanosomes*

No oxygen uptake was observed with a reaction mixture containing 0·4 ml. mouse or rat serum and 0·02M glucose in modified Ringer–phosphate solution. Therefore, no correction was applied to experiments in which serum was added.

Washed trypanosomes were suspended in 0·02M glucose in Ringer–phosphate solution, and 0·1 ml. mouse serum was added to some flasks. The oxygen uptake of the trypanosomes in the presence of serum was compared directly with the oxygen uptake in the absence of serum.

From Table 4 it can be seen that the oxygen uptake of washed *T. lewisi* or *T. equiperdum* in the presence of serum was greater than in the absence of serum.

Table 1. *The effect of mouse serum or blood on the oxygen uptake of trypanosomes*

(The average O_2 uptake is expressed as $\mu l.O_2/10^8$ trypansomes/hr. for the periods 10–70 and 70–130 min. or 0–60 and 60–120 min. after the beginning of the experiment. Extreme values are given below the average figure. The O_2 uptake in the 2nd hour is also expressed as a percentage of the O_2 uptake in the 1st hour, with the number of experiments given in parentheses.)

* $4–5 \times 10^7$ trypanosomes per flask gave 48 %. $2–3 \times 10^7$ trypanosomes per flask gave 66 %.

	T. lewisi, 3 to 4-day			*T. lewisi*, 7 to 9-day			*T. equiperdum*		
	O_2 uptake $\mu l.$		% O_2 uptake 70–130 min.	O_2 uptake $\mu l.$		% O_2 uptake 70–130 min.	O_2 uptake $\mu l.$		% O_2 uptake 70–130 min.
Trypanosome preparation	10–70 min.	70–130 min.	10–70 min.	10–70 min.	70–130 min.	10–70 min.	10–70 min.	70–130 min.	10–70 min.
Heparinized infected rat blood	73·4	56·0	76	105	88	83·8	149	86	48* 66*
+ 0·02 M glucose	71–76	54–57	(3)	85–124	70–100	(5)	137–157	68–101	(4)
Washed trypanosomes	52·1	43·1	82·8	87·4	74·8	85·6	130	52	40
+ 0·02 M glucose	35–70	24–60	(4)	71–99	65–84	(9)	110–139	35–78	(9)
				0–60 min.	60–120 min.	% 60–120 min. / 0–60 min.	0–60 min.	60–120 min.	% 60–120 min. / 0–60 min.
Washed trypanosomes	—	—	—	89	76·5	86	138	56·7	41
+ 0·02 M glucose				73–103	65–85	(14)	120–160	36–78	(13)
Washed typanosomes	—	—	—	100	89	89	156	108·5	69·6
+ 0·02 M glucose				89–111	83–98	(5)	147–170	92–124	(11)
+ 11·1 % mouse serum									
Washed trypanosomes	—	—	—	97·1	80·6	83	154·8	104·2	67·4
+ 0·02 M glucose				89–105	72–89	(5)	145–160	97–115	(6)
+ 22·2 % mouse blood									

(3) *The effect of mouse blood on the oxygen uptake of washed trypanosomes*

Washed trypanosomes were suspended in 0·02M glucose in Ringer–phosphate solution, and 0·2 ml. heparinized normal mouse blood was added to some flasks. Using this preparation, it was possible to compare directly the oxygen uptake of the trypanosomes in the presence and absence of blood, and to include control flasks containing 0·2 ml. of the same heparinized mouse blood in Ringer–phosphate–glucose solution in each experiment. The oxygen uptake of these control flasks was deducted from that of the experimental flasks containing blood.

In several experiments, the oxygen uptake of washed trypanosomes in the presence of 0·2 ml. mouse blood was compared with the oxygen uptake in the presence of 0·1 ml. mouse serum. When the correction for the oxygen uptake of the blood was deducted, it was found that the oxygen uptake in the presence of blood was very similar to that in the presence of serum.

One difference between *T. lewisi* and *T. equiperdum* can be seen by comparing the oxygen uptake of the non-inhibited controls shown in Figs. 5, 6, 10 and 11. With *T. lewisi*, the oxygen uptake in the presence of blood or serum commenced at a higher rate than with Ringer–phosphate–glucose solution. The rate of oxygen uptake decreased slowly in all three instances and the rate in the second hour of experiment was 86% of that in the first hour with Ringer–phosphate–glucose solution, 83% in the presence of blood and 89% in the presence of serum.

That is, blood or serum increased the oxygen uptake of *T. lewisi* by a constant amount throughout experiments lasting 2 hr. Trypanosomes in Ringer–phosphate–glucose solution were still very active at the end of experiments lasting 2 hr., and there was little difference in motility between them and trypanosomes in solutions containing blood or serum.

With *T. equiperdum*, the oxygen uptake in the presence of blood or serum commenced at almost the same rate as with Ringer–phosphate–glucose solution. The rate of oxygen uptake decreased rapidly in the absence of blood or serum, so that the oxygen uptake in the second hour of experiment was 41% of that in the first hour. In the presence of mouse blood or serum, the rate of oxygen uptake did not decrease so rapidly, and the oxygen uptake in the second hour of experiment was 67·4 and 69·6% of that in the first hour, respectively. This compares well with the figure of 66% found with heparinized infected blood when the pH did not fall below 7·3. The pH in flasks containing serum was similar to that in flasks where it was omitted.

Blood or serum had, therefore, little effect on the oxygen uptake of *T. equiperdum* at the beginning of experiments, but maintained the oxygen uptake at a higher rate during the second hour of experiments. At the end of 2 hr., the trypanosomes were more active in the flasks containing blood or serum than in those with Ringer–phosphate–glucose solution.

Differences in oxygen uptake in the presence and absence of blood have previously been given for several species of trypanosomes.

T. gambiense had an O_2 uptake of 178 μl./10^8 trypanosomes/hr. (over 50 min.)

in infected heparinized blood, but only 133 μl. when suspended in serum–Ringer-glucose solution (von Brand, Weinbach & Tobie, 1955).

T. gambiense had an O_2 uptake of 161 μl./10^8 trypanosomes/hr when suspended in saline plus blood, compared with 113 μl. when suspended in saline plus serum (von Brand, Tobie, Mehlman & Weinbach, 1953).

T. equiperdum had an O_2 uptake of 108 μl./10^8 trypanosomes/hr. (over 40 min.) in infected defibrinated blood, compared with 91 μl. when suspended in serum–Ringer–glucose solution (Agosin & von Brand, 1954).

When *T. evansi* was separated from the blood and suspended in glucose-buffer solution, the trypanosomes had only 50 % of the O_2 uptake of the whole blood suspension in phosphate–citrate–saline solution. Nearly 75 % of the lost activity was restored by adding 5 % mouse plasma (Marshall, 1948).

T. lewisi suspended in Ringer–phosphate–glucose solution had only 65 % of the oxygen uptake of trypanosomes in citrated infected blood (over 2 hr.). When suspended in Ringer–phosphate–glucose with serum, the oxygen uptake of the trypanosomes was raised to 74 % of that in citrated infected blood. That is, an oxygen uptake of approximately 75 μl./10^8 trypanosomes/hr. was recorded in Ringer–phosphate–glucose solution, 85 μl. in Ringer–phosphate–glucose–serum solution, and 115 μl. in citrated infected blood (Moulder, 1948*a*). The comparisons between oxygen uptake in the presence and absence of blood or serum found in the present investigation agree with those shown above when such factors as differences in the method of preparing suspensions are taken into account.

Using *T. equiperdum*, Reiner, Smythe & Pedlow (1936) found that trypanosomes maintained a higher rate of oxygen uptake over 2 hr. when suspended in Ringer-phosphate solution in the presence of serum than they did in the absence of serum. I have found no other figures showing the rate of decrease of oxygen uptake during the course of experiments, with which to compare the results given in the present paper.

IV. THE EFFECT OF IODOACETIC ACID ON OXYGEN UPTAKE

(1) *The effect of iodoacetic acid on normal rat blood*

Preliminary experiments showed that concentrations of iodoacetic acid lower than 10^{-3}M had no effect on the oxygen uptake of normal rat blood during experiments lasting 90 min., but that 10^{-3}M and higher concentrations caused an increase in oxygen uptake. Therefore, concentrations greater than $3{\cdot}3 \times 10^{-4}$M were not tested against trypanosomes when blood was included in the reaction mixture.

The oxygen uptake of 0·6 ml. aliquots of heparinized rat blood in Ringer-phosphate–glucose solution was determined as described above in §III (1). Iodoacetic acid was tipped into the reaction mixture 10 or 15 min. after manometric readings were begun and readings were continued for up to $4\frac{1}{2}$ hr.

Fig. 1 shows the result of one experiment with normal rat blood and 10^{-3}M and $3{\cdot}3 \times 10^{-2}$M iodoacetic acid. The readings at 15 min. varied between 6 and 11 μl. The average figure has been plotted for all the curves to make comparisons easier. In the flasks containing 10^{-3}M iodoacetic acid, the oxygen uptake of the

blood began to increase after 50 min. and continued for a further 3½ hr. In the flasks containing $3{\cdot}3 \times 10^{-2}$M iodoacetic acid, the rate of oxygen uptake decreased slightly at first but began to increase after 50 min. This increase was more rapid than with 10^{-3}M iodoacetic acid, but continued for only a further 2 hr. and the rate of

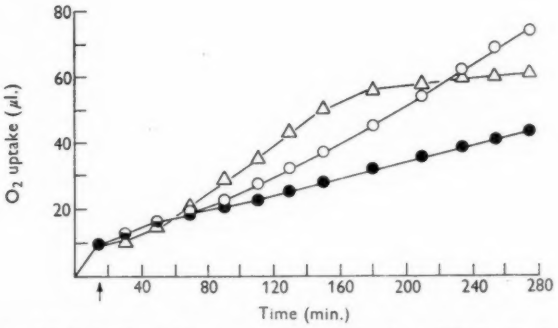


Fig. 1. The effect of iodoacetic acid on normal rat blood at 37° C. Each flask contained 0·6 ml. heparinized rat blood in Ringer–phosphate–glucose solution. Iodoacetic acid was added 15 min. after manometric readings were begun. ●—●, Heparinized rat blood; ○—○, heparinized rat blood + 10^{-3}M iodoacetic acid; △—△, heparinized rat blood + $3{\cdot}3 \times 10^{-2}$M iodoacetic acid.

oxygen uptake was less than in the control flasks during the final hour of the experiment. In this experiment, another sample of iodoacetic acid was neutralized with NaOH and a 10^{-3}M solution of this neutralized iodoacetic acid gave a curve that was identical with the 10^{-3}M iodoacetic acid. In another experiment, iodoacetic acid was again neutralized with NaOH and $3{\cdot}3 \times 10^{-2}$M neutralized iodoacetic acid gave the same result as $3{\cdot}3 \times 10^{-2}$M iodoacetic acid, although the pH of the reaction mixture remained at 7·5 while that with the acid dropped to 7·35.

Iodoacetic acid (10^{-3}M) was then tested with rat blood in Ringer–phosphate–glucose solution in the presence of 2×10^{-4}M methylene blue and the results are shown in Fig. 2. Methylene blue increased the oxygen uptake of the rat blood very considerably. When it was present, 10^{-3}M iodoacetic acid inhibited oxygen uptake, although the same concentration increased the oxygen uptake in control flasks containing the same rat blood without methylene blue.

When Michaelis & Salomon (1930) showed the effect of methylene blue on oxygen uptake of blood cells, they used washed rabbit blood cells resuspended in the same volume of buffered Ringer solution. In the present investigation, a further sample of the same heparinized rat blood was washed three times in Ringer–phosphate–glucose solution and resuspended in Ringer–phosphate–glucose solution to restore the original volume. The previous experiment with 10^{-3}M iodoacetic acid and 2×10^{-4}M methylene blue was then repeated using the suspension of washed blood cells instead of whole blood. This second experiment was begun 2 hr. after the first one began. The results are shown in Fig. 2. The results were very similar to those with the whole blood preparations: iodoacetic acid caused inhibition in the

presence of methylene blue, and increased oxygen uptake in its absence. The inhibition of the washed blood cells was greater than the inhibition of the whole blood preparation. An initial rapid rate of oxygen uptake was observed with the whole blood preparation, both in the presence and in the absence of methylene blue: this initial rapid rate was absent when washed blood cells were used, and oxygen uptake continued at a constant rate.

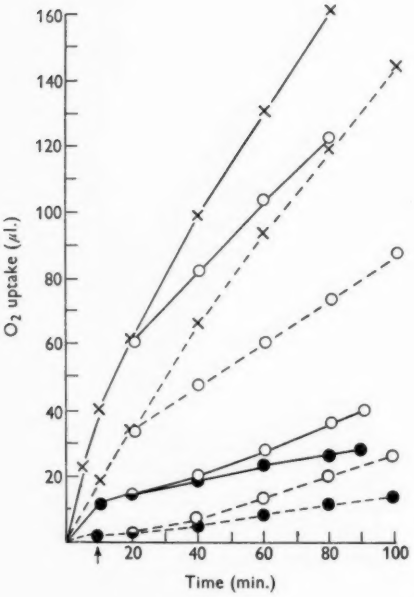


Fig. 2

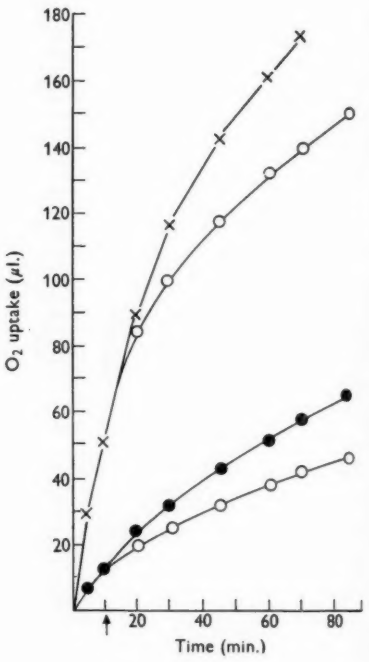


Fig. 3

Fig. 2. The effect of iodoacetic acid on rat blood or washed blood cells at 37° C in the presence of methylene blue. Each flask contained 0·6 ml. heparinized rat blood, ———, or 0·6 ml. washed blood cells, – – –, in Ringer–phosphate–glucose solution. Flasks ×—× and ×– –× also contained $2{\cdot}0 \times 10^{-4}$ M methylene blue. Flasks ●—● and ●– – –● contained no methylene blue. 10^{-3} M iodoacetic acid was added 10 min. after manometric readings were begun, in flasks ○—○ and ○– – –○.

Fig. 3. The effect of iodoacetic acid on rat blood cells which had been treated with sodium nitrate to convert haemoglobin into methaemoglobin. Each flask contained 0·6 ml. washed methaemoglobin rat blood cells in Ringer–phosphate–glucose solution at 37° C. Flask ×—× also contained $2{\cdot}0 \times 10^{-4}$ M methylene blue. Flask ●—● contained no methylene blue. 10^{-3} M iodoacetic acid was added 10 min. after manometric readings were begun in flask ○–○.

Suspensions of rat blood cells in which the haemoglobin had been converted into methaemoglobin were then tested with 10^{-3} M iodoacetic acid. Heparinized blood was treated with half its volume of isotonic (1·06 %) sodium nitrite solution and left for 20 min. The blood cells were centrifuged and washed once with sodium nitrite solution, washed three times with Ringer–phosphate solution and resuspended in Ringer–phosphate–glucose solution to restore the original volume. The previous

experiment was then repeated, using 10^{-3}M iodoacetic acid and 2×10^{-4}M methylene blue. Two hours elapsed between taking the blood from the heart of the rat and putting the manometer flasks into the water-bath. Fig. 3 shows that iodoacetic acid inhibited oxygen uptake by the suspension of methaemoglobin blood cells, both in the presence and in the absence of methylene blue.

(2) *The effect of iodoacetic acid on the oxygen uptake of* Trypanosoma lewisi

Iodoacetic acid was tested against *T. lewisi* by von Brand *et al.* (1950), using defibrinated blood preparations. There was only slight inhibition of oxygen uptake. Ryley (1956) showed that oxygen uptake was inhibited to a much greater extent when iodoacetic acid was tested against washed trypanosomes suspended in Ringer–phosphate–glucose solution than when it was tested against heparinized infected blood. These results are included in Table 2.

In the present investigation, several concentrations of iodoacetic acid were tested against *T. lewisi* in heparinized infected blood or against washed *T. lewisi* suspended in a variety of substrates. The results are included in Table 2; and Figs. 4–8 show the effect of $3{\cdot}3 \times 10^{-4}$M iodoacetic acid on the O_2 uptake of the trypanosomes. A summary of the effect of iodoacetic acid in the presence of various substrates is given below.

Heparinized infected blood with glucose (Fig. 4 and Table 2). Each flask contained 0·6 ml. heparinized infected blood and 0·02M glucose in Ringer–phosphate solution. Oxygen uptake was very slightly reduced in the presence of 10^{-4}M or $3{\cdot}3 \times 10^{-4}$M iodoacetic acid.

Glucose (Fig. 5 and Table 2). The oxygen uptake of washed trypanosomes in Ringer–phosphate solution containing 0·02M glucose was inhibited strongly by iodoacetic acid.

Glucose plus serum (Fig. 6 and Table 2). The effect of iodoacetic acid on the oxygen uptake of washed trypanosomes in Ringer–phosphate solution containing 0·02M glucose and 11·1% freshly prepared mouse serum was intermediate between the effect on trypanosomes in heparinized infected blood and trypanosomes in Ringer–phosphate–glucose solution.

Glucose plus blood (Table 2). When washed trypanosomes were suspended in Ringer–phosphate solution containing 0·02M glucose and 22·2% freshly prepared rat blood, 10^{-4}M iodoacetic acid caused less inhibition of oxygen uptake than when serum alone was present.

Glutamine (Fig. 7 and Table 2). Iodoacetic acid very slightly reduced the oxygen uptake of washed trypanosomes suspended in Ringer–phosphate solution containing 0·005M glutamine. The inhibition was similar to that with heparinized infected blood.

Glutamine plus glucose (Table 2). When the reaction mixture contained 0·02M glucose and 0·005M glutamine, $3{\cdot}3 \times 10^{-4}$M iodoacetic acid caused very slight inhibition of oxygen uptake. This degree of inhibition was similar to that found in the presence of glutamine alone.

Glucosamine (Fig. 8 and Table 2). When washed trypanosomes were suspended in Ringer–phosphate solution containing 0·02M glucosamine, $3{\cdot}3 \times 10^{-4}$M iodoacetic

Table 2. *The effect of iodoacetic acid on the oxygen uptake of* Trypanosoma lewisi *in the presence of various substrates*

(The O_2 uptake of trypanosomes inhibited by specified concentrations of iodoacetic acid is expressed as a percentage of non-inhibited O_2 uptake. In the present investigation, the O_2 uptake was measured for 60 min. after the addition of the inhibitor, and 3–7 experiments were performed with each concentration of inhibitor except in those cases marked with asterisks where only 2 experiments were performed. The average percentage O_2 uptake is recorded and extreme values are given below. Symbols for motility: −, all trypanosomes immotile; (+), occasional trypanosomes moving; +, decreased motility; ++, normal or very slightly decreased motility. Mot = motility.)

Trypanosome preparation	Substrate	Author	Concentration of iodoacetic acid: 10^{-2}M %O_2	Mot.	5×10^{-3}M %O_2	Mot.	$3{\cdot}3 \times 10^{-3}$M %O_2	Mot.	10^{-3}M %O_2	Mot.	$3{\cdot}3 \times 10^{-4}$M %O_2	Mot.	10^{-4}M %O_2	Mot.	10^{-5}M %O_2	Mot.
Defibrinated blood	Glucose	v. Brand, Tobie & Mehlman (1950)	.	.	35	(+)	.	.	66	++	92	++	.	.	90	++
Heparinized blood		Ryley (1956)	.	.	.	.	82	.	90	.	98	.	90	.	98	.
Heparinized blood	Glucose	.	.	.	.	.	.	.	.	.	93	++	97*	++	.	.
											89–96		96–98			
Washed trypanosomes	Glucose	Moulder (1948*a*)	1	.	.	.	.	.	.	.	.	.	.	.	.	.
Washed trypanosomes	Glucose	Ryley (1956)	.	.	.	.	7	.	10	.	17	.	42	.	.	.
Washed trypanosomes	Glucose	.	.	.	.	.	.	.	19	−	29	−	26	−	.	.
									14–21		18–36		22–30			
Washed trypanosomes	Glucose +serum	.	.	.	.	.	.	.	47	+	.	.	57*	+	.	.
									44–52				56–57			
Washed trypanosomes	Glucose +blood	.	.	.	.	.	.	.	.	.	.	.	76	++	.	.
													71–83			
Washed trypanosomes	Glutamine	.	.	.	.	.	.	.	66*	+	85	++	93	++	.	.
									58–74		72–96		86–98			
Washed trypanosomes	Glucose +glutamine	.	.	.	.	.	.	.	.	.	84*	++	.	.	.	.
											80–88					
Washed trypanosomes	Glucosamine	.	.	.	.	.	.	.	.	.	48*	(+)	.	.	.	.
											46–50					
Washed trypanosomes	Glycerol	.	.	.	.	.	.	.	.	.	33	−	.	.	.	.
											31–34					

acid caused less inhibition of oxygen uptake than when glucose was the substrate; inhibition was however, much greater than when glutamine was the substrate.

Glycerol (Table 2). The oxygen uptake of washed trypanosomes suspended in Ringer–phosphate solution with 0·02 M glycerol was inhibited by $3{\cdot}3 \times 10^{-4}$ M iodoacetic acid to the same extent as when the substrate was glucose.

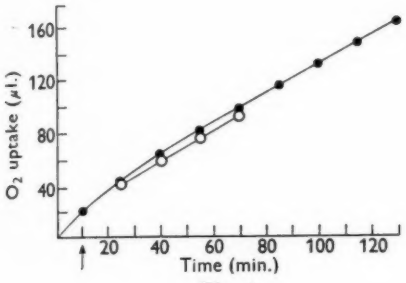


Fig. 4

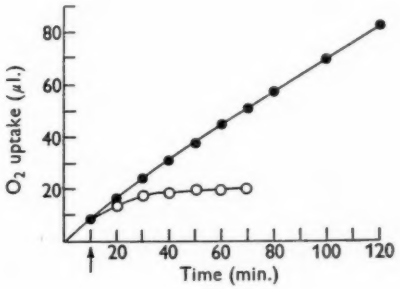
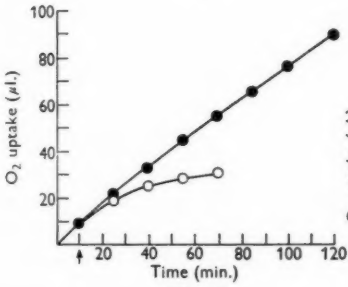


Fig. 5



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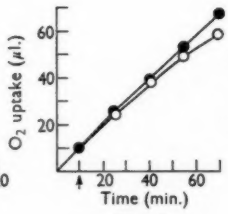

Fig. 7

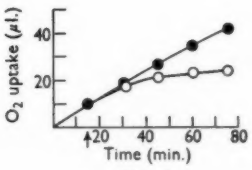

Fig. 8

Figs. 4–8. The effect of $3{\cdot}3 \times 10^{-4}$ M iodoacetic acid on the oxygen uptake of *T. lewisi* at 37° C. in the presence of various substrates. Each flask is represented as containing $5{\cdot}0 \times 10^7$ trypanosomes in Ringer–phosphate solution. In actual experiments there were between 4·0 and $5{\cdot}6 \times 10^7$ trypanosomes per flask. Iodoacetic acid was added 10 or 15 min. after manometric readings were begun. ●—●, Non-inhibited oxygen uptale; ○—○, oxygen uptake with iodoacetic acid.

Fig. 4. Oxygen uptake of 0·6 ml. heparinized rat blood infected with *T. lewisi*.

Fig. 5. *T. lewisi* in Ringer–phosphate solution with 0·02 M glucose.

Fig. 6. *T. lewisi* in Ringer–phosphate solution with 0·02 M glucose and 11·1 % mouse serum.

Fig. 7. *T. lewisi* in Ringer–phosphate solution with 0·005 M glutamine.

Fig. 8. *T. lewisi* in Ringer–phosphate solution with 0·02 M glucosamine.

(3) *The effect of iodoacetic acid on the oxygen uptake of* Trypanosoma equiperdum

Iodoacetic acid was tested against *T. equiperdum* by von Brand *et al.* (1950), using defibrinated blood preparations. This trypanosome was more sensitive than *T. lewisi* to the inhibitor. Ryley (1956) found that the degree of inhibition was similar with heparinized infected blood and with trypanosomes suspended in Ringer–phosphate–glucose solution. These results are included in Table 3. In

addition, Marshall (1948) with *T. evansi*, and Harvey (1949) with *T. hippicum* obtained approximately 50% inhibition with $3{\cdot}58 \times 10^{-6}$M and $1{\cdot}6 \times 10^{-6}$M iodoacetic acid respectively. These figures indicate a much greater sensitivity than was found in the present series.

In the present investigation, several concentrations of iodoacetic acid were tested against *T. equiperdum* in heparinized infected blood, or against washed *T. equiperdum* suspended in a variety of substrates. The results are included in Table 3; and Figs. 9–12 show the effect of 10^{-5}M iodoacetic acid. A summary of the effect of iodoacetic acid in the presence of various substrates is given below.

Heparinized infected blood with glucose (Fig. 9 and Table 3). Each flask contained 0·6 ml. heparinized infected blood and 0·02M glucose in Ringer–phosphate solution. Oxygen uptake was only very slightly reduced in the presence of 10^{-5}M iodoacetic acid, but with 10^{-4} M iodoacetic acid it was reduced to 28% of that of non-inhibited controls.

Glucose (Fig. 10 and Table 3). The oxygen uptake of washed trypanosomes suspended in Ringer–phosphate solution containing 0·02M glucose was inhibited strongly by iodoacetic acid.

Glucose plus serum (Fig. 11 and Table 3). Iodoacetic acid was tested against washed *T. equiperdum* suspended in Ringer–phosphate–glucose solution with 11·1% freshly prepared mouse serum. The degree of inhibition with 10^{-4}M and $3{\cdot}3 \times 10^{-4}$M iodoacetic acid was similar to that in the absence of serum; but in the presence of serum the trypanosomes were less sensitive to the action of 10^{-5}M iodoacetic acid than when suspended in Ringer–phosphate–glucose solution.

Glucose plus blood (Table 3). The difference noted above with serum was even more marked when the mouse serum was replaced by 22·2% freshly prepared heparinized mouse blood. The degree of inhibition with 10^{-4}M and $3{\cdot}3 \times 10^{-4}$M iodoacetic acid was similar to that in the absence of blood; but in the presence of blood 10^{-5}M iodoacetic acid caused only very slight inhibition of oxygen uptake.

Glycerol (Fig. 12 and Table 3). The oxygen uptake of washed trypanosomes suspended in Ringer–phosphate solution containing 0·02M glycerol was inhibited slightly by 10^{-5}M iodoacetic acid. With stronger concentrations of iodoacetic acid, the inhibition was considerably less than had been found with the other substrates.

Glucose plus glycerol (Table 3). The results with 0·02M glucose plus 0·02M glycerol as substrate varied according to the concentration of iodoacetic acid that was tested. With 10^{-4}M iodoacetic acid, the inhibition with glucose plus glycerol resembled that with glucose alone. With 10^{-5}M iodoacetic acid, the inhibition was less than with glucose, although more than with glycerol as the only substrate.

V. THE EFFECT OF ARSENICALS ON THE OXYGEN UPTAKE OF *TRYPANOSOMA LEWISI* AND *T. EQUIPERDUM*

Experiments by von Brand *et al.* (1950) with trypanosomes in defibrinated blood showed that mapharsen and sodium arsenite caused greater inhibition of the oxygen uptake of *T. equiperdum* than of *T. lewisi*. Ryley (1951) found that sodium arsenite

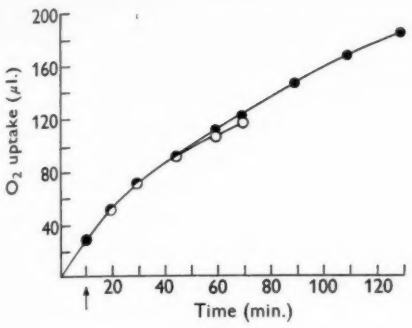

Fig. 9

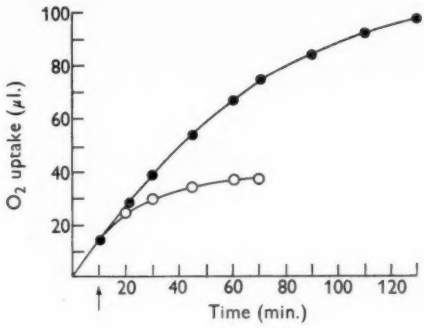

Fig. 10

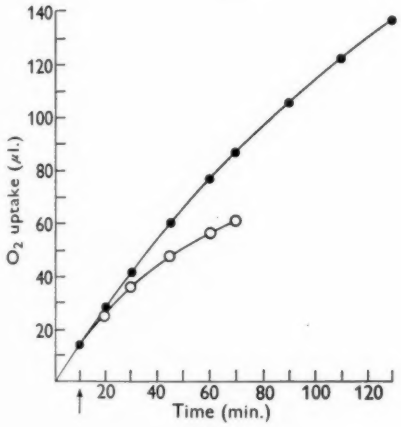

Fig. 11

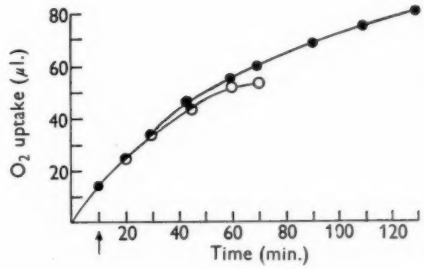

Fig. 12

Figs. 9–12. The effect of 10^{-5} M iodoacetic acid on the oxygen uptake of *T. equiperdum* at 37° C in the presence of various substrates. Each flask is represented as containing $5{\cdot}0 \times 10^7$ trypanosomes in Ringer–phosphate solution. In actual experiments, there were $2{\cdot}9 \times 10^7$ trypanosomes per flask in Fig. 9 and between $4{\cdot}0$ and $4{\cdot}4 \times 10^7$ trypanosomes per flask in Figs. 10–12. Iodoacetic acid was added 10 min. after manometric readings were begun. ●—●, non-inhibited oxygen uptake; ○—○, oxygen uptake with iodoacetic acid.

Fig. 9. Oxygen uptake of 0·6 ml. heparinized rat blood infected with *T. equiperdum*.

Fig. 10. *T. equiperdum* in Ringer–phosphate solution with 0·02 M glucose.

Fig. 11. *T. equiperdum* in Ringer–phosphate solution with 0·02 M glucose and 11·1 % mouse serum.

Fig. 12. *T. equiperdum* in Ringer–phosphate solution with 0·02 M glycerol.

caused a comparable degree of inhibition of oxygen uptake when washed *T. lewisi* were suspended in Ringer–phosphate–glucose solution. These results are included in Table 4. Marshall (1948), working with *T. evansi*, found that $1{\cdot}98 \times 10^{-5}$ M phenylarsine oxide reduced oxygen uptake to 18·7 % of that of non-inhibited controls, $3{\cdot}97 \times 10^{-6}$ M to 66 %, and $9{\cdot}93 \times 10^{-7}$ M to 85·4 %.

In the present experiments, neoarsphenamine was tested against washed *T. lewisi* suspended in Ringer–phosphate solution containing glucose or glutamine,

Table 3. *The effect of iodoacetic acid on the oxygen uptake of* Trypanosoma equiperdum *in the presence of various substrates*

(The O_2 uptake of trypanosomes inhibited by specified concentrations of iodoacetic acid is expressed as a percentage of non-inhibited O_2 uptake. The symbols for motility, etc., are the same as those given in Table 2.)

			Concentration of iodoacetic acid													
			$3{\cdot}3 \times 10^{-3}$ M		10^{-3} M		$3{\cdot}3 \times 10^{-4}$ M		2×10^{-4} M		10^{-4} M		$3{\cdot}3 \times 10^{-5}$ M		10^{-5} M	
Trypanosome preparation	Substrate	Author	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.
Defibrinated blood	Glucose	v. Brand *et al.* (1950)	.	.	4	−	.	.	15	+	34	++	.	.	95	++
Heparinized blood	.	Ryley (1956)	16	.	35	.	39	.	.	.	48	.	74	.	.	.
Heparinized blood	Glucose	.	.	.	.	.	19 16–23	−	.	.	28 21–35	(+)	56 37–67	+	97	++
Washed trypanosomes	Glucose	Ryley (1956)	24	.	27	.	33	.	.	.	67	.	100	.	.	.
Washed trypanosomes	Glucose	.	.	.	.	.	10 8–12	−	.	.	18 9–21	−	24 19–38	−	37 33–40	−
Washed trypanosomes	Glucose + serum	.	.	.	.	.	12 8–17	−	.	.	17 14–22	−	32 26–36	−	67 57–80	(+)
Washed trypanosomes	Glucose + blood	.	.	.	.	.	.	.	.	.	14 11–19	−	33 27–39	−	94 90–96	++
Washed trypanosomes	Glycerol	.	.	.	.	.	27 22–34	−	.	.	45 36–65	−	64 55–78	−	86 79–94	+
Washed trypanosomes	Glucose + glycerol	.	.	.	.	.	.	.	.	.	14 11–18	−	.	.	68 62–74	(+)

and against washed *T. equiperdum* suspended in Ringer–phosphate solution containing glucose or glycerol. The results are shown in Table 4. Neoarsphenamine inhibited the oxygen uptake of *T. equiperdum* more than that of *T. lewisi*. The effect on *T. lewisi* when glutamine was present was similar to that when glucose was present as substrate. Inhibition of the oxygen uptake of *T. equiperdum* was slightly less when glycerol was present than when glucose was present. This difference between the two substrates was not as pronounced as the difference observed with iodoacetic acid.

The trivalent arsenical compound, reduced stovarsol thioglycollate was also tested against *T. equiperdum*. As is shown in Table 4, this inhibitor was less active than neoarsphenamine. This is probably partly because each molecule of stovarsol contains one atom of arsenic, whereas each molecule of neoarsphenamine contains two atoms of arsenic. Neoarsphenamine is probably hydrolysed almost immediately because it acted at least as quickly as the trivalent compound, as is shown in Figs. 13 and 14. Reduced stovarsol thioglycollate inhibited oxygen uptake to almost the same extent when glycerol was present as when glucose was present as substrate.

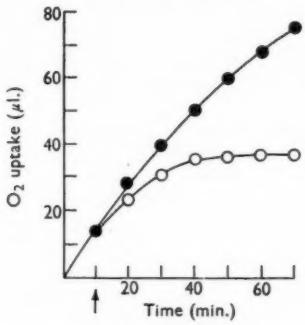

Fig. 13

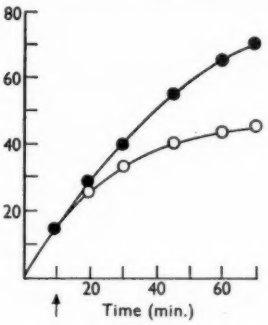

Fig. 14

Fig. 13. The effect of $3{\cdot}3 \times 10^{-5}$ M neoarsphenamine on the oxygen uptake of *T. equiperdum* at 37° C. in Ringer–phosphate–glucose solution. Each flask is represented as containing $5{\cdot}0 \times 10^7$ trypanosomes. In actual experiments, there were $4{\cdot}4 \times 10^7$ trypanosomes per flask. Neoarsphenamine was added 10 min. after manometric readings were begun. ●—●, O_2 uptake of non-inhibited trypanosomes; ○—○, O_2 uptake of trypanosomes in the presence of neoarsphenamine.

Fig. 14. The effect of 10^{-4} M reduced stovarsol thioglycollate on the oxygen uptake of *Trypanosoma equiperdum* at 37° C. in Ringer–phosphate–glucose solution. Each flask is represented as containing $5{\cdot}0 \times 10^7$ trypanosomes. In actual experiments there were $5{\cdot}5 \times 10^7$ trypanosomes per flask. Reduced stovarsol thioglycollate was added 10 min. after manometric readings were begun. ●—●, O_2 uptake of non-inhibited trypanosomes; ○—○, O_2 uptake of trypanosomes in the presence of reduced stovarsol thioglycollate.

Sodium arsenite was also tested against *T. equiperdum*. As is shown in Table 4, this inhibitor was as active as reduced stovarsol thioglycollate when glucose was present. However, in the presence of glycerol, 10^{-4} M sodium arsenite had little effect on the oxygen uptake within the first hour, although the oxygen uptake subsequently decreased considerably and the trypanosomes became non-motile. In this delay of action in the presence of glycerol, sodium arsenite differed from the two organic arsenicals.

Table 4. *The effect of arsenicals on the oxygen uptake of* Trypanosoma lewisi *and* T. equiperdum *in the presence of various substrates*

(The O_2 uptake of trypanosomes inhibited by specified concentrations of the arsenicals is expressed as a percentage of non-inhibited O_2 uptake. The symbols for motility, etc., are the same as those given in Table 2.)

				Concentration of inhibitor											
				$2{\cdot}0 \times 10^{-4}$ M		$3{\cdot}3 \times 10^{-4}$ M		10^{-4} M		$3{\cdot}3 \times 10^{-5}$ M		$2{\cdot}0 \times 10^{-5}$ M		10^{-5} M	
Trypanosome preparation	Substrate	Inhibitor	Author	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.	$\%O_2$	Mot.
T. lewisi															
Defibrinated blood	Glucose	Mapharsen	v. Brand *et al.* (1950)	59	++	.	.	83	++	.	.	87	++	84	++
Defibrinated blood	Glucose	Arsenite	v. Brand *et al.* (1950)	46	+	.	.	51	+	.	.	81	++	87	++
Washed trypanosomes	Glucose	Arsenite	Ryley (1951)	.	.	.	.	41	.	.	.	.	.	.	.
Washed trypanosomes	Glucose	Neoarsphenamine	.	.	.	35 30–38	−	73 70–76	+	94 92–96	++	.	.	.	.
Washed trypanosomes	Glutamine	Neoarsphenamine	.	.	.	24* 23–25	−	65* 62–68	+	92* 91–93	++	.	.	.	.
T. equiperdum															
Defibrinated blood	Glucose	Mapharsen	v. Brand *et al.* (1950)	5	−	.	.	7	−	.	.	32	(+)	43	+
Defibrinated blood	Glucose	Arsenite	v. Brand *et al.* (1950)	7	−	.	.	6	−	.	.	69	++	84	++
Washed trypanosomes	Glucose	Neoarsphenamine	.	.	.	.	.	22 15–29	−	36 28–50	−	.	.	66 65–68	(+)
Washed trypanosomes	Glycerol	Neoarsphenamine	.	.	.	.	.	39 33–47	−	52 40–62	−	.	.	75 70–82	(+)
Washed trypanosomes	Glucose	Arsenite	.	.	.	.	.	56 53–60	−	70 67–73	+	.	.	.	.
Washed trypanosomes	Glycerol	Arsenite	.	.	.	.	.	96 94–98	+	.	.	.	.	.	.
Washed trypanosomes	Glucose	Reduced stovarsol thioglycollate	.	.	.	.	.	60 52–66	−	.	.	.	.	.	.
Washed trypanosomes	Glycerol	Reduced stovarsol thioglycollate	.	.	.	.	.	64 56–72	−	.	.	.	.	.	.

VI. DISCUSSION

T. lewisi was considerably more sensitive to the action of iodoacetic acid when the substrate was glucose than when it was glutamine. That is, the inhibitor probably acts primarily against some part of the glycolytic chain; probably against the enzyme triose phosphate dehydrogenase as suggested by Marshall (1948). It is more difficult to analyse the difference found with *T. equiperdum* between the strong inhibition caused by iodoacetic acid in the presence of glucose and the slight inhibition in the presence of glycerol, because it is not known how the metabolism of glycerol is linked to that of glucose.

With both *T. lewisi* and *T. equiperdum*, iodoacetic acid was more active against washed trypanosomes suspended in Ringer–phosphate–glucose solution than against trypanosomes in the presence of blood. As glutamine and a number of other amino-acids increase the oxygen uptake of *T. lewisi* (Moulder, 1948*a*; Thurston, 1957), it was thought that the trypanosomes might have utilized these substances, which are present in the blood, when glucose metabolism was inhibited. However, *T. equiperdum* does not appear to use amino-acids as a source of energy and so some other factor must account for the effect of the blood.

T. lewisi was as sensitive to the action of neoarsphenamine when glutamine was the substrate as when glucose was the substrate. That is, the inhibitor does not act solely against some part of the glycolytic chain. Marshall (1948) suggested that phenylarsine oxide acted mainly on the hexokinase enzyme of *T. evansi*, because ATP accumulated and intermediates behind the block were rapidly consumed. In the present experiments, neoarsphenamine and reduced stovarsol thioglycollate were almost as active when glycerol was the substrate as when glucose was the substrate, and so hexokinase cannot be the only enzyme affected. Sodium arsenite acted differently from the other two compounds and may affect hexokinase specifically.

VII. SUMMARY

1. The oxygen uptake of *Trypanosoma lewisi* and *T. equiperdum* was measured with differential manometers using microflasks of 5 ml. capacity. Each flask contained $2–5 \times 10^7$ trypanosomes.

2. The oxygen uptake of trypanosomes in heparinized infected blood was compared with that of washed trypanosomes suspended in Ringer–phosphate–glucose solution with or without added serum or blood. With *T. lewisi*, the oxygen uptake began at a higher rate in the presence of blood or serum than with Ringer–phosphate–glucose solution. With *T. equiperdum*, the oxygen uptake in the presence of blood or serum began at almost the same rate as with Ringer–phosphate–glucose solution, but the rate of oxygen uptake decreased more rapidly in the absence of blood or serum than when it was present.

3. High concentrations of iodoacetic acid (e.g. $3{\cdot}3 \times 10^{-2}$ M) caused an increase in oxygen uptake when tipped into heparinized normal rat blood. Similar concentrations caused an inhibition of oxygen uptake when $2{\cdot}0 \times 10^{-4}$ M methylene

blue was also present or when the blood had been treated with sodium nitrite to convert haemoglobin into methaemoglobin.

4. Iodoacetic acid caused little inhibition of oxygen uptake when *T. lewisi* was suspended in Ringer–phosphate solution containing glutamine or glutamine plus glucose, or when heparinized infected blood was tested. Inhibition was greatest when the substrate was glucose, while glucosamine, glycerol, or glucose plus serum gave intermediate results.

5. Iodoacetic acid caused least inhibition of oxygen uptake when *T. equiperdum* was suspended in Ringer–phosphate solution containing glycerol, or when heparinized infected blood was tested. Inhibition was greatest when the substrate was glucose, and was slightly less marked when the substrate was glucose plus serum or glucose plus glycerol.

6. Neoarsphenamine inhibited the oxygen uptake of *T. lewisi* to a similar extent whether glucose or glutamine was present.

7. Neoarsphenamine and reduced stovarsol thioglycollate inhibited the oxygen uptake of *T. equiperdum* slightly less when the substrate was glycerol than when it was glucose. Sodium arsenite was as active as reduced stovarsol thioglycollate when the substrate was glucose, but 10^{-4}M sodium arsenite had little effect when the substrate was glycerol.

This work was carried out with the aid of a grant from the Agricultural Research Council. I should like to thank Professor D. Keilin and Dr P. Tate for helpful discussions, and Dr F. Hawking of the National Institute for Medical Research for his gift of reduced stovarsol thioglycollate.

REFERENCES

AGOSIN, M. & BRAND, T. VON (1954). The influence of puromycin on the carbohydrate metabolism of *Trypanosoma equiperdum*. *Antibiotics & Chemotherapy*, **4**, 624–32.

BRAND, T. VON & TOBIE, E. J. (1948). Further observations on the influence of cyanide on some trypanosomes. *J. cell. comp. Physiol.* **31**, 49–68.

BRAND, T. VON, TOBIE, E. J. & MEHLMAN, B. (1950). The influence of some sulfhydryl inhibitors and of fluoroacetate on the oxygen consumption of some trypanosomes. *J. cell. comp. Physiol.* **35**, 273–300.

BRAND, T. VON, TOBIE, E. J., MEHLMAN, B. & WEINBACH, E. C. (1953). Observations on the metabolism of normal and arsenic-resistant *Trypanosoma gambiense*. *J. cell. comp. Physiol.* **41**, 1–22.

BRAND, T. VON, WEINBACH, E. C. & TOBIE, E. J. (1955). Comparative studies on the metabolism of the culture form and bloodstream form of *Trypanosoma gambiense*. *J. cell. comp. Physiol.* **45**, 421–33.

HARVEY, S. C. (1949). The carbohydrate metabolism of *Trypanosoma hippicum*. *J. biol. Chem.* **179**, 435–53.

MARSHALL, P. B. (1948). The glucose metabolism of *Trypanosoma evansi* and the action of trypanocides. *Brit. J. Pharmacol.* **3**, 8–14.

MICHAELIS, L. & SALOMON, K. (1930). Respiration of mammalian erythrocytes. *J. gen. Physiol.* **13**, 683–93.

MOULDER, J. W. (1948*a*). The oxidative metabolism of *Trypanosoma lewisi* in a phosphate-saline medium. *J. infect. Dis.* **83**, 33–41.

MOULDER, J. W. (1948*b*). Changes in the glucose metabolism of *Trypanosoma lewisi* during the course of infection in the rat. *J. infect. Dis.* **83**, 42–9.

REINER, L., SMYTHE, C. V. & PEDLOW, J. T. (1936). On the glucose metabolism of trypanosomes (*Trypanosoma equiperdum* and *Trypanosoma lewisi*). *J. biol. Chem.* **113**, 75–88.

RYLEY, J. F. (1951). Studies on the metabolism of the Protozoa. 1. Metabolism of the parasitic flagellate, *Trypanosoma lewisi*. *Biochem. J.* **49**, 577–85.

RYLEY, J. F. (1956). Studies on the metabolism of the Protozoa. 7. Comparative carbohydrate metabolism of eleven species of trypanosome. *Biochem. J.* **62**, 215–22.

THURSTON, J. P. (1957). The effect of oxygen uptake of *Trypanosoma lewisi* and *Trypanosoma equiperdum*, with special reference to oxygen consumption in the presence of amino-acids. *Parasitology*, **48**, 149–64.

(*MS. received for publication* 13. VIII. 1957.—Ed.)

OBSERVATIONS ON THE DEVELOPMENT OF *TOXOCARA CANIS* (WERNER, 1782) IN THE DOG*

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(With Plate IX and 19 Figures in the Text)

CONTENTS

I. Introduction 184
II. Methods 187
III. Observations on naturally infected dogs in Brisbane . 188
IV. Experimental infections 190
V. Development of larvae 195
VI. Discussion 205
VII. Summary 206
References 208

I. INTRODUCTION

Throughout the nineteenth century it was generally accepted that two species of ascaridoid nematode occurred in dogs and cats, namely '*Ascaris marginata*' in dogs and '*Ascaris mystax*' in cats; some authorities considered that only a single species, '*A. mystax*', occurred in both dogs and cats. This has led to some confusion, e.g. Nelson (1852) studied the early development of '*A. mystax*' from dogs, but his illustrations reveal that his material was *Toxascaris leonina*. Development of larvae within the egg of '*Ascaris marginata*' from dogs was observed by Verloren (1854, cited by Davaine, 1863), but it is difficult to determine to what species these observations referred.

Hering (1873) concluded from a survey in Stuttgart that dogs under 6 months old usually harboured '*A. marginata*' and that older dogs were less often infected and harboured fewer worms. He found larvae in the intestine of a 6-day-old puppy, and observed that they increased in size and number over the first 6 weeks; eggs were found in the intestine on the 17th day. Finding no worms in newborn puppies (he did not examine the lungs), but finding hair and bedding material in the stomach 12 days after birth, he concluded that infection of puppies occurred by the ingestion of eggs adherent to the teats and derived from worms in the bitch, rather than by parental infection. He attempted to infect puppies by feeding eggs, but his results were inconclusive.

Subsequent to the clarification of the species of ascaridoid parasites occurring in dogs and cats by Railliet & Henry (1911) the identity of the species concerned in life-history studies could be established. The nomenclature of these parasites has been recently reviewed by Sprent (1956), and it is clear that two species occur in dogs, namely, *Toxascaris leonina* (= *T. limbata*) and *Toxocara canis* (= *Belascaris marginata*). Hering's observations indicate that the parasites he found in very

* An abstract of this paper has already been published (*J. Parasitol.* **43**, suppl. p. 45).

young puppies were *T. canis*. In older puppies (between 6 weeks and 3 months), he observed that the worms were of different sizes, and concluded accordingly that re-infection must have taken place, but it is possible that finding worms of variable size in the older puppies was due to a mixed infection with *T. canis* and *Toxascaris leonina*.

Fülleborn (1921) demonstrated that the puppies born of a bitch, which he had infected during pregnancy by subcutaneous injection of larvae derived from *Toxocara canis*, were heavily infected with *T. canis* at birth, thus indicating that prenatal infection had occurred. In the puppies killed just after birth, larvae were found in the liver and lungs; in a puppy killed 2 days later there were moulting larvae in the lungs and in the intestine; at 6 days, the larvae in the intestine had reached a length of about 4 mm., and at 16 days they measured 20–30 mm.

Prenatal infection was again demonstrated by Shillinger & Cram (1923), who administered embryonated eggs by the mouth to a bitch in advanced pregnancy. Eight of her twelve puppies died at birth and four died on the following day; six had larvae in the liver, one had larvae in the lungs and one had larvae in both liver and lungs; the larvae measured 900–950μ in length. No larvae were found in the intestine of the bitch. Hadley, Warwick & Gildow (1924) examined newborn fox cubs and found larvae in the lungs; they measured about 1·07 mm. in length. No larvae were found in the intestine.

Augustine (1927) infected a bitch with eggs 33 days after coitus; her puppies were born 27 days later. No larvae were found in three puppies which were killed at birth, but in a day-old puppy there were larvae in the bronchi (1–1·2 mm. long), and in a 6-day-old puppy there were larvae in the stomach (7 mm. long) and in the intestine (3–6 mm. long). The bitch had two small worms in the intestine. Similarly, he infected a second bitch, and her puppies were born 33 days later. At birth there were larvae in the liver (0·7–0·9 mm. long), at 1 day there were larvae in the lungs (1 mm. long), at 2 days there were larvae in the lungs (1 mm. long) and by 3 days the larvae in the lungs had grown to a length of 1·5–2 mm. At 6 days the larvae had reached the intestine, they measured 2–4 mm. At 3 weeks young adults were present. There were no worms in the bitch.

Petrov (1941) found *T. canis* in both young and adult silver foxes, the former were more frequently and more heavily infected. He found that 27 % of stillborn fox cubs harboured migrating larvae; these were found in the blood, liver and lungs. He observed that they reached the intestine within the first few days of life, although larvae could still be found in the lungs several days after birth. Among dogs kept under experimental conditions, Nifontov (1949) found that 97·7 % were infected with *T. canis* by 1 month of age and 89·7 % of newborn pups were infected at birth.

It has been assumed that prenatal infection with *T. canis* results from embryonated eggs which have been ingested during pregnancy. Yutuc (1949) reported a case where a bitch was treated five times before service, and, except for exercise in a clean concrete pen, was confined during pregnancy in a cage which was cleaned daily. The pups were not allowed to come in contact with the floor, and the teats of the bitch were frequently washed. Nevertheless, three out of her six puppies

harboured *T. canis* within 27 days of birth. Yutuc suggested that larvae ingested before gestation may accumulate in the tissues of the bitch, and during pregnancy invade the foetuses. The presence of larvae in the kidneys of adult dogs and foxes reported by several observers (Fülleborn, 1921; Schmid, 1934; Petrov, 1941) may be of significance in this connexion.

Table 1. *Host list for* Toxocara canis*

† Syn: *Belascaris triquetra* (Schrank, of Rudolphi) Leiper, 1907.
B. marginata (Rudolphi) Railliet & Henry, 1911.
B. vulpis (Froelich) Railliet & Henry, 1911.
B. masculior Railliet & Henry, 1911.
Toxocara vulpis (Froelich) Yorke & Maplestone, 1926.
T. masculior (Railliet & Henry, 1911) Yorke & Maplestone, 1926.

Host	Common name	Author
[*Alopex*] *lagopus*	Arctic fox	McIntosh (correspondence)‡
A. beringensis	—	Petrov, 1930
Canis mexicanus	Grey wolf	Canavan, 1931
C. lupus	Wolf	Canavan, 1929
C. occidentalis	Northern timber wolf	Cowan 1947
C. dingo	Dingo	Canavan, 1931
C. aureus	Jackal	Baylis & Daubney, 1922
C. familiaris (type host)	Dog	Railliet & Henry, 1911
?*C. pallipes*	Indian wolf	Baylis & Daubney, 1922
Crocuta crocuta	Spotted hyaena	Mozgovoi, 1953
Fennecus zerda	Fennec fox	Railliet & Henry 1911
Lycaon pictus (=*capensis*)	Cape hunting dog	Vevers, 1923
Nyctereutes procyonoides ussuriensis	Raccoon dog	Mozgovoi, 1953
[*Urocyon*] *cinereoargenteus Scotti*	Scott's grey fox	Canavan, 1931
Vulpes bengalensis	Indian fox	Baylis & Daubney, 1922
[*V. fulva*] *pennysylvanicus*	American red fox	Canavan, 1931
V. fulva regalis	Northern plains red fox	Smith, 1943
V. velox	Swift fox	Canavan, 1929
V. vulpes	European fox	Vevers, 1923
V. vulpes japonicus	—	Yamaguti 1941

* Also reported in cats by Hitchcock (1953), in *Felgis tigris* and *Acinonyx jubatus* by Kreis (1952), in *Procyon lotor* and *Lynx rufa* by Mozgovoi (1953), and in *Meles anakuma* by Yamaguti (1941).

† Names used before Leiper (1907) cannot be used in synonymy unless differentiation from *Toxascaris* can be established.

‡ Identified by Mr A. McIntosh, United States Department of Agriculture, Beltsville, Md., from a collection of worms, B.A.I. no. 4145 labelled '*Ascaris canis*', from '*Vulpes lagopus*'.

? indicates a doubtful record.

[] indicates that an earlier name was used by the author.

Yamaguti (1941) described *Toxocara tanuki* from *Nyctereutes procyonoides viverrinus*.

The evidence that prenatal infection with *T. canis* may occur in both dogs and silver foxes appears to be conclusive; but it is uncertain to what extent infection may also occur in postnatal life. The occurrence of *T. canis* in adult dogs, foxes and other Canidae (Table 1) indicates that postnatal infection occurs, but it is also possible that some of the worms acquired prenatally may survive and persist for years in the host. If postnatal infection occurs, it would be logical to suppose that

such animals become infected by ingestion of eggs, or by ingestion of some intermediate host harbouring the second-stage larvae in the tissues. Both these methods of infection have been demonstrated in the cat with *T. cati* by Sprent (1956).

In relation to *T. canis*, the former method has been tried by several investigators with variable success. Fülleborn (1921) attempted to infect dogs directly by feeding the embryonated eggs. He administered eggs to a newborn puppy and 13 days later found larvae 4 mm. long in the intestine, but in older dogs infected in this way no worms appeared in the intestine, although larvae were found in nodules in the liver, lungs and kidneys. Petrov (1941) fed *T. canis* eggs to two fox pups, $2\frac{1}{2}$ months old, and found mature worms 29 days later; two puppies which he had not infected harboured no worms. Shoho (1955) reported that no infection resulted after feeding several puppies, 40 days old, with eggs of *T. canis*.

Infection of dogs by ingesting tissues of animals harbouring the larvae of *T. canis* was suggested by the work of Fülleborn (1921), Matoff (1949) and Hoeppli, Feng, & Li (1949) who showed that the larvae of *T. canis* persist in the tissues of mice. This mode of infection was successfully demonstrated by Petrov (cited by Mozgovoi, 1953) in foxes, and Sprent (1953) in dogs. Sprent (1952) showed that in experimentally infected mice, the migratory pathway of the larvae of *T. canis* as well as certain other species occurring in carnivorous animals, differed from that of the larvae of *Ascaris suum* and *Parascaris equorum*, in that the former were distributed to various tissues and remained there (somatic migration) whereas the latter, after passing through the lungs, reached the alimentary tract and passed out in the faeces (tracheal migration). Furthermore, it was shown (Sprent, 1953) that the larvae of *Toxocara canis* remained alive in mouse tissues for 6 months and could survive several weeks in a frozen carcass.

Nichols (1956) found that the length of larvae of *T. canis* pressed from eggs was 0·360–0·434 mm., while the length of larvae recovered from mouse tissues, irrespective of duration of infection, was 0·357–0·445 mm. These results indicate that the larvae undergo no growth while in the tissues of mice; similar findings were reported by Tiner (1953), Beaver, Snyder, Carrera, Dent & Lafferty (1952) and Sprent (1955). Nichols (1956) described the second-stage larva of *T. canis* from eggs and from the tissues of mice, but there appears to be no published description of the further development of the larvae of *T. canis* in dogs. The purpose of the present paper is to report some observations on the development of this parasite, as observed in natural infections and in infections produced by feeding dogs with embryonated eggs and with infected mice.

II. METHODS

Eggs were collected from the uterus of female worms and cultured as previously described (Sprent, 1956). They were incubated at room temperature and were not used for infection until at least 28 days old. Mice were infected and the larvae collected from their tissues as previously described (Sprent, 1952). Dogs were infected with eggs by mixing them with minced meat; infected mouse tissues were

minced and mixed with meat. The dogs were kept in closed kennels with concrete floors; they were fed raw meat and milk once daily. Experimental work with *T. canis* in dogs is rendered difficult because of the high incidence of infection with this parasite in newborn puppies. It was therefore necessary in some experiments, especially those in which dogs were fed infected mouse tissues, to treat the animal with an anthelmintic drug before infection. The time, dose and anthelmintic drug used, is indicated under the particular experiment (see below). Two drugs were used, diethylcarbamazine acid citrate (Banocide) and piperazine adipate (Entacyl).

The tissues of dogs were searched for larvae by two methods: (1) by digestion of the minced organ with 1 % pepsin at pH 1 at 37° C. for 6–18 hr, the time depending upon the bulk of tissue to be digested. In the case of puppies the whole carcass after removal of the viscera, was minced and digested, but in adult dogs portions of muscle and connective tissue were removed from the neck trunk and limbs, and digested; this is referred to below as 'muscle'. After digestion and sedimentation, the material was fixed by adding an equal quantity of boiling 6 % formalin; (2) by placing the minced organ over muslin suspended over a beaker of physiological saline at 37° C. for 4–6 hr. The sediment was fixed as above. The contents of the trachea, oesophagus, stomach and intestine were obtained by slitting open in physiological saline, pulling the wall between the blades of forceps and fixing the washing as above.

After fixation small amounts of the material were searched with the 32 mm. objective in a round dish constructed to fit the mechanical stage. The whole of the fixed material was thus searched and the larvae picked out with a fine pipette into 5 % glycerin in 70 % alcohol. After evaporation the larvae were mounted in pure glycerin and the cover-slip sealed with finger-nail varnish. When the number of larvae was small, they were all mounted in this way for examination and measurement, but when more than thirty larvae were collected, after finding the range of length measurements, twenty to thirty larvae were picked at random for mounting. Measurements were made by means of a camera lucida and fine fuse wire, and in all cases refer to glycerin-mounted larvae.

III. OBSERVATIONS ON NATURALLY INFECTED DOGS IN BRISBANE

Dogs of various ages were examined at autopsy for the presence of *T. canis*. The findings were as follows:

(*a*) *Puppies* (1–3 *weeks*)

Foetuses near term (D6). Six puppies were removed at Caesarian section: the heart, liver, lungs, brain and kidneys of each were removed and digested together. Two third-stage larvae (length 0·94 and 0·83 mm.) were found in the combined hearts and nineteen third-stage larvae (length 0·88–1·1 mm.) in the combined lungs. No larvae were found in the alimentary tract.

Two hours (DP808). Carcass, liver and lungs were digested. Twenty-two larvae (length 0·41–0·71 mm.) were found in the liver, they appeared to be degenerated and it was not possible to determine their stage of development by their structure. There were no larvae in the alimentary tract.

Twelve hours (DP896). The liver and lungs were chopped up finely and placed over muslin in physiological saline at 37° C. Five third-stage larvae (length 0·76–1·0 mm.) were collected from the lungs.

Fourteen hours (D43). The liver and lungs were digested. Twenty-two third-stage larvae were found in the lungs (length 0·74–1·08 mm.). There were no larvae in the alimentary tract.

One day (D59). The carcass, liver and lungs were digested. One third-stage larva (length 0·85 mm.) was found in the carcass, two-third-stage larvae (length of one 0·90 mm.) in the lungs, and two third-stage larvae (length of one 0·85 mm.) in the stomach.

Two days (D70/D71). The carcass, liver, lungs and kidneys of two puppies from the same bitch were digested. Twenty third-stage larvae (length 0·60–1·26 mm.) were found in the lungs. There were no larvae in the alimentary tract.

Two days (D37). The liver and lungs were digested and a single third-stage larva (length 1·0 mm.) was found in the lungs. No larvae were found in the alimentary tract.

Three days (D44). In the intestinal contents were ten fourth-stage larvae (length 2·3–3·7 mm.). Other tissues were not examined.

Five days (DP914). Lungs were minced and placed over muslin in physiological saline at 37° C. One third-stage larva (length 1·25 mm.) and one fourth-stage larva (length 1·7 mm.) were found in the lungs. Two fourth-stage larvae (length 2·0 and 2·5 mm.) were found in the intestine.

Six days (DP916). Examined as DP914. One fourth-stage larva (length 2·4 mm.) was found in the intestine.

Seven days (D73). The carcass, liver and lungs were digested. Fifteen larvae were found in the lungs, but some were damaged; of those intact two were in the third-stage (length 0·62 and 0·98 mm.), two were moulting (length 1·2 and 1·3 mm.), five were in the fourth stage (length 1·5–2·0 mm.). There were no larvae in the intestinal contents.

Eight days (DP924–926). Three puppies from the same litter were examined as DP914. In the stomach and intestine, fourteen fourth-stage larvae (length 3·1–7·0 mm.) were found in the first puppy; in the second were twenty-two fourth-stage larvae (length 5·5–7·1 mm.); in the third were eight fourth-stage larvae (length 2·9–5·8 mm.). Several were moulting.

Eleven days (DP919). Examined as DP914. Two adults (length of one 9·8 mm.) were found in the intestine.

Eleven days (DP920). Examined as DP914. One fourth-stage larva (length 3·18 mm.) and one adult (length 6·8 mm.) were found in the stomach, four adults (length 7·7–11·3 mm.) were found in the intestine.

Thirteen days (D16). The intestine contained twenty-eight adult worms (length 12–20 mm.). Lungs were not examined.

Fourteen days (D78). In the intestinal contents were seventy-nine adult worms (length 8·5–30 mm.). Other tissues were not examined.

Sixteen days (DP917). Examined as DP914. Two adults (length 9 and 35 mm.) were found in the stomach and eight adults (length 28–40 mm.) were found in the intestine.

Seventeen days (DP917). Examined as DP914. Six adults (length 28–53 mm.) were found in the intestine.

Twenty-one days (D82, 83, 85, 88). In the stomach and intestinal contents of four puppies from the same litter there were 355, 147, 166, 207 worms respectively. Their lengths ranged from 3·1 to 66·6 mm.

(*b*) *Puppies* (1–6 *months*)

The intestinal contents of twenty-nine puppies were examined and all were found to harbour mature *T. canis*.

(*c*) *Adult dogs* (*over* 6 *months*)

The intestinal contents of twenty-nine adult dogs were examined, and three were found to be infected with *T. canis*. In these three infections the number of worms recovered was 4, 1 and 1 respectively. In one of these dogs, white spots were observed on the kidneys, resembling those seen on the kidneys of dogs experimentally infected with eggs of *T. canis*; these were found to contain living second-stage larvae (length 0·40–0·43 mm.).

The above observations indicated that in natural prenatal infection of puppies with *T. canis*, third-stage larvae are already in the lungs at birth. As no second-stage larvae were found, it appears probable that the second moult occurs before birth. It was observed that third-stage larvae grow in the lungs to a length of approximately 1 mm. By the third day of life, some of the larvae had reached the intestine and attained the fourth stage, although for several days after birth third-stage larvae were still to be found in the lungs. By the end of the first week immature adults were present in the intestine. The incidence of infection was found to be much higher in dogs under 6 months old.

IV. EXPERIMENTAL INFECTIONS

(1) *Mice*. A series of fourteen mice were given 5000 eggs of *T. canis*. They were killed 1–14 days after infection and the various tissues were digested and searched for larvae. Distribution of the larvae is shown in Table 2. It will be observed that this distribution confirms previous reports (Matoff, 1949; Beaver *et al.* 1952; Tiner, 1953; Sprent, 1952, 1955) that the larvae of *T. canis* undergo a predominantly somatic migration and appear in large numbers in the carcass and brain of mice.

(2) *Dogs*. The experiments recorded below were designed to discover whether dogs can be infected (*a*) by oral administration of embryonated eggs, and (*b*) by ingestion of an intermediate host, such as the mouse.

(*a*) *Observations on dogs infected by oral administration of embryonated eggs of* Toxocara canis

(1) An adult dog (D21) was infected with 10,000 eggs and killed 8 days later. The liver, brain, kidneys and lungs were digested. Sixteen second-stage larvae (length 0·34–0·38 mm.) were found in the liver; no larvae were found in the stomach and intestine.

(2) An adult dog (D7) was infected with 3000 eggs and killed 8 days later. The liver, lungs, kidneys, brain and heart were digested. Thirteen second-stage larvae (length 0·36–0·40 mm.) were found in the liver, fourteen in the lungs (length 0·36–0·40 mm.), seven in the kidney (length 0·38–0·40 mm.), and two in the brain (length 0·38 and 0·39 mm.). No larvae were found in the stomach and intestine.

Table 2. *Distribution of* Toxocara canis *larvae in mice after infection with* 5000 *eggs*

Days after infection	Mouse no.	Lungs	Intestinal and stomach wall	Small intestine and stomach contents	Kidney	Carcass	Liver	Brain
1	2333	1	478	0	1	13	500	0
2	2334	58	15	0	35	52	1402	14
3	2335	545	27	0	47	237	498	37
4	2315	380	0	0	38	340	121	74
5	2316	—	32	1	119	574	539	63
6	2317	346	20	4	51	608	99	413
7	2319	26	9	0	20	680	59	252
8	2326	9	1	0	15	310	17	176
10	2320	27	2	1	11	545	20	365
11	2321	1	3	0	1	206	5	157
12	2322	10	2	0	0	663	17	384
13	2323	5	1	0	4	337	28	225
14	2324	5	8	0	1	750	32	633

(3) An adult dog (D8) was given a large number of eggs (actual number not recorded) and killed 8 days later. The lungs, liver, kidneys and brain were digested. Nine second-stage larvae (length 0·38–0·41 mm.) were found in the liver, five in the lungs (length 0·38–0·40 mm.), twenty in the kidneys (length 0·38–0·42) and one in the brain (length 0·40 mm.). No larvae were found in the stomach and intestine.

(4) An adult dog (D34) was infected with 3000 eggs and killed 9 days later. The lungs, kidneys and brain were digested. One second-stage larva was found in the lungs, one in the liver, and one in the kidney. No larvae were found in the stomach and intestine.

(5) A 5-week puppy (D15) was given 2500 eggs and killed 8 days later. The liver, lungs, brain, heart and kidneys were digested. Larvae (not examined) were found in the liver and lungs, but there were no larvae in the stomach and intestine.

(6) A puppy (D68), aged 6 weeks, was kept for 2 months in a concrete pen. Its faeces were examined weekly for the presence of *T. canis*, but none was observed, indicating an absence of prenatal infection. It was given three doses of 10,000 eggs at weekly intervals and killed 1 week after the last dose. The following tissues were digested and searched for larvae; the liver, lungs, brain, heart, kidneys, perirenal connective tissues, uterus, perirectal connective tissue, neck muscles, lumbar muscles, thigh muscles, stomach wall and intestinal wall. The contents of the

trachea, stomach and intestine were washed out and searched for larvae. Thirty-two second-stage larvae were recovered from the lungs (length 0·36–0·41 mm.), in some of these the cuticle was loose at the anterior end; thirty-two were recovered from the kidneys (length 0·34–0·42 mm.) and three from the lumbar muscles (length 0·36–0·41 mm.). There were no larvae in the stomach and intestine.

At autopsy, dogs infected with eggs (Expts. 1–6) showed red spots on the lungs, pale streaks and spots on the liver, and white spots on the kidneys. The larvae recovered from these dogs were almost of uniform size (length 0·34–0·42 mm.), there was no evidence of development beyond the second-stage, and although the cuticle appeared loose in some instances, no actual moulting forms were observed. No larvae were recovered from the alimentary tract.

(7) Four newborn puppies (D1–D4) were infected by oral administration of 1000 *T. canis* eggs. They were killed at 3, 9, 16 and 23 days after infection respectively. The liver, lungs, kidneys, heart and brain were digested. At 3 days (D1) there were seventy-one second-stage larvae in the liver (length 0·36–0·43 mm.), thirty in the lungs (length 0·39–0·41 mm.) and seven in the heart muscle (length 0·37–0·44 mm.). They resembled those observed in the tissue of older dogs infected by eggs. Several of these larvae showed a loosening of the cuticle, and in the heart one larva (length 0·44 mm.) was moulting. In the intestinal contents there was a single fourth-stage larva (length 3·0 mm.); this larva was probably derived from a natural prenatal infection (cf. D44). At 9 days (D2) there were four second-stage larvae (length 0·37–0·39 mm.) and one moulting second-stage larva (length 0·44 mm.) in the liver. In the lungs there was one second-stage larva (length 0·39 mm.), and two third-stage larvae (length 0·69–0·90 mm.). In the stomach contents there were ninety-three larvae at different stages of growth (length from 0·48 to 0·83 mm.). In a sample of twenty, eighteen of the larvae were in the third stage; there was also a moulting third-stage larva (length 1·2 mm.) and a fourth-stage larva (length 1·5 mm.). In the intestinal contents there were two moulting third-stage larvae (length of both 1·3 mm.), two fourth-stage larvae (length 1·66 and 2·05 mm.), and two adults (length 15 and 12 mm.); the latter were probably derived from prenatal infection (cf. DP920). At 16 days (D3) there were three small larvae in the lungs, but they were damaged. In the intestinal contents there were eight fourth-stage larvae (length 2·6–6·3 mm.), six moulting fourth-stage (length 5·5–7·4 mm.), ninety-three immature adults (length 7·6–15 mm.), and one larger immature adult (length 37 mm.) which probably represented a prenatal infection (cf. DP917). At 23 days (D4) there were twenty-three fourth-stage larvae (length 2·5–5·5 mm.), four moulting fourth-stage larvae (length 5·8–7·1 mm.), and several hundred immature adults (length 6·3–51 mm.) in the intestinal contents; two adult females containing eggs (length 89 and 97 mm.) were also present, they probably represented a prenatal infection (cf. D82–88).

It appeared likely that two infections were present in these puppies: (*a*) a prenatal infection, represented by the fourth-stage larva in the intestine of D1, the two immature adults in the intestine of D2, the single larger immature adult in the intestine of D3, and the two mature adults in the intestine of D4 (cf. the previously described observations on natural infections in puppies of this age);

(*b*) a more intensive superimposed infection of younger larvae which were probably derived from the experimental infection with eggs.

(8) From a litter of six puppies, one puppy was killed daily for the first 4 days of life. The liver, lungs and contents of the alimentary tract of these four puppies were searched to detect the extent of prenatal infection. This was found to be slight, as shown by the fact that there were no larvae in the livers; there were six, two, five, and no third-stage larvae respectively in the lungs, and there were no larvae in the alimentary tract. Eight days after birth one puppy (D76) was given 5000 eggs of *T. canis* orally. It was killed 9 days later. The larvae were counted as follows: two third-stage larvae (length 0·55 and 0·91 mm.) in the liver; 175 larvae* (length 0·59–0·97 mm.) in the lungs, two were moulting (length 0·98 and 1·0 mm.); one second-stage larva (length 0·41 mm.) in the muscle; 329 larvae* (length 0·46–0·89 mm.) in the stomach wall; 159 larvae* (length 0·47–0·97 mm.) in the stomach contents. There were also eight adults (lengths 23–24 mm.) in the intestine, which probably represented a prenatal infection (cf. DP917). Another puppy (D77) was given 5000 eggs at 19 days after birth and was killed 9 days later. There was one damaged larva (length 0·45 mm.) in the stomach wall, one damaged larva (length 0·42 mm.) in the intestinal wall. In the stomach contents there were two second-stage larvae (length 0·41 and 0·43 mm.), three third-stage larvae (length 0·83 and 0·97 mm.), one moulting third-stage larva (length 1·1 mm.), and one fourth-stage larva (length 1·3 mm.). In the intestinal contents there were two moulting fourth-stage larvae (length 5·4 and 6·3 mm.), one fourth-stage larva (length 5·3 mm.) and one adult (length 5·9 mm.). There were also four larger adults (length 14–20 mm.) which probably represented a prenatal infection, although no explanation can be given as to why these adults were smaller than those found in D76.

These experiments demonstrated that oral administration of embryonated eggs to dogs of 5 weeks and older resulted in the presence of second-stage larvae in the tissues; no further development of these larvae was observed and no larvae were found in the alimentary tract. On the other hand, similar infection of four newborn puppies, one 8-day-old puppy and one 19-day-old puppy indicated that development proceeded beyond the second stage and that intestinal infection could be produced by this method of infection. The number of larvae reaching the alimentary tract was considerably smaller in the 19-day-old puppy than in the younger puppies.

(*b*) *Observations on dogs infected by ingestion of tissues of infected mice*

(1) A 5-week-old puppy (D14) was treated with 100 mg. 'Banocide' and 2 days later was infected by feeding tissues of two mice (1376 and 1377) infected previously with *T. canis* eggs. It was killed 10 days later. Four fourth-stage larvae were found in the intestine (length 2·2–3·1 mm.).

(2) Five puppies, which were born on 21 March, were treated on 20 April, 6 May, 19 May and 22 May, with 100 mg. of 'Banocide'. Three days after the last

* In samples of twenty larvae, all were found to be in the third stage.

treatment, two puppies (D28 and D31) were given mice (1486 and 1487) which had been infected with *T. canis* 25 days previously, and two puppies (D29 and D32) were given mice which had been infected with *T. cati* (1470 and 1471) 42 days previously. Two puppies were left as controls (D33 and D30). Three days later D28 and D31 were again given mice from the same series infected with *T. canis*, and D29 and D32 were again given mice infected with *T. cati*. Six days later (3 June) D29 (*T. cati*) was killed: two adult *T. canis* were found in the intestine, but there were no larvae. D32 was given three further infections at 3-day intervals, with mouse tissues infected with *T. cati*, and was left for 4 weeks, the faeces being examined weekly for eggs; none was observed and the dog was discarded. D31 was killed on 4 June and the stomach was found to contain ten third-stage larvae (length 0·59–1·0 mm.). D28 was given three further infections of mouse tissues infected with *T. canis*, at 3-day intervals, and was killed on 16 June. The intestine contained one mature adult, fourteen immature adults (length 5·9–10 mm.) and ten fourth-stage larvae (length 1·2–5·0 mm.). One control (D33) died on 26 May, the other (D30) was killed on 17 June; the former contained no larvae or adults, but in the latter one adult was found.

(3) Three puppies (D64–D66), 5 months old, which were already naturally infected with *T. canis* were given 600 mg. 'Banocide'. Seventeen days later each puppy was given minced tissues of two mice, which had been infected with 5000 eggs of *T. canis* 44 days previously. Three puppies were killed at 3, 5 and 7 days after infection. The liver, lungs, kidneys, muscle, intestinal wall and stomach wall were digested, and the contents of the stomach and small intestine were searched for larvae. No larvae were found, except for one second-stage larva in the liver and one second stage larva in the lungs of the puppy killed at 5 days.

(4) Two puppies (D73A and D74), 7 weeks old, were treated with 600 mg. 'Entacyl' and 11 days later were each given two mice which had been infected 16 days previously with 5000 *T. canis* eggs. One (D73A) was killed 3 days after infection, and the liver, lungs, kidney, muscle, stomach wall and intestinal wall were digested. One second-stage larva (length 0·36 mm.) was found in the liver; fifteen second-stage larvae (length 0·39–0·43 mm.) were found in the stomach wall, two of them were moulting (length 0·41 and 0·43 mm.). There was one immature adult (length 9·2 mm.) in the intestine. The other (D74) was killed 6 days after infection, two larvae (lost) were found in the kidney and one second-stage larva (length 0·40 mm.) was found in the stomach wall; there were no larvae in the stomach and intestinal contents.

(5) One 7-week-old puppy (D75) was treated with 600 mg. 'Entacyl'. Twenty-one days later it was given two mice infected 26 days previously with 5000 *T. canis* eggs. It was killed 9 days after infection. The liver, lungs, kidney, muscle, stomach wall and intestinal wall were digested; no larvae were found. In the intestine there were eleven fourth-stage larvae (length 2·6–5·3 mm.). There were also three mature males which had evidently not been removed by anthelmintic treatment.

(6) Two fox pups (F1–F2), aged about 6 weeks, were given minced tissues of two mice which had been infected 3 weeks previously with 5000 eggs of *T. canis*. Two other fox pups (F3–F4) from the same litter were given minced tissues of

2 mice which had been infected 2 weeks previously with 5000 eggs of *T. cati*. The first pair were killed at 17 (F 1) and 21 (F 2) days after infection. One of the second pair (F 3) died 8 days after infection, the other (F 4) was killed 17 days after infection. The liver, lungs, kidneys, muscle, stomach wall and stomach contents and intestinal contents were searched for larvae.

In F 1 there were eighteen second-stage larvae (length 0·34–0·37 mm.) in the lungs, several were moulting (length 0·37–0·45 mm.); there was also one third-stage larva (length 0·51 mm.). In the stomach wall there were two damaged second-stage larvae. In the intestinal wall there was one second-stage larva (length 0·36 mm.). In the stomach contents there was one second-stage larva (length 0·42 mm.), one third-stage larva (length 0·56 mm.) and two fourth-stage larvae (length 3·7 and 5·2 mm.). In the intestinal contents there were seven fourth-stage larvae (length 3·1–5·4 mm.), and thirty-four immature adults (length 5·8–7·6 mm.).

In F 2 there were two second-stage larvae in the liver, nineteen second-stage larvae in the lungs, five second-stage larvae in the kidney, and two second-stage larvae in the intestinal wall; all these larvae measured between 0·35 and 0·40 mm. Several larvae in the lungs had loose cuticle, but appeared to be damaged, one larva in the lungs had an oral prolongation similar to that described in *T. cati* larvae (Sprent, 1956). In the stomach contents there was a third-stage larva with loose cuticle (length 1·2 mm.) and a fourth-stage larva (length 4·5 mm.). In the intestine there were three fourth-stage larvae (length 3·0–5·8 mm.) and twenty-eight immature adults (length 6·9–15 mm.). In F 3 there were no larvae in any of the tissues examined. In F 4 there was one larva in the lung (length 0·35 mm.), two in the muscle (length of one 0·4 mm.) and one degenerated fourth-stage larva in the stomach contents (length 5·1 mm.); presumably these were *T. cati* larvae, but it was not found possible to differentiate them from *T. canis* larvae.

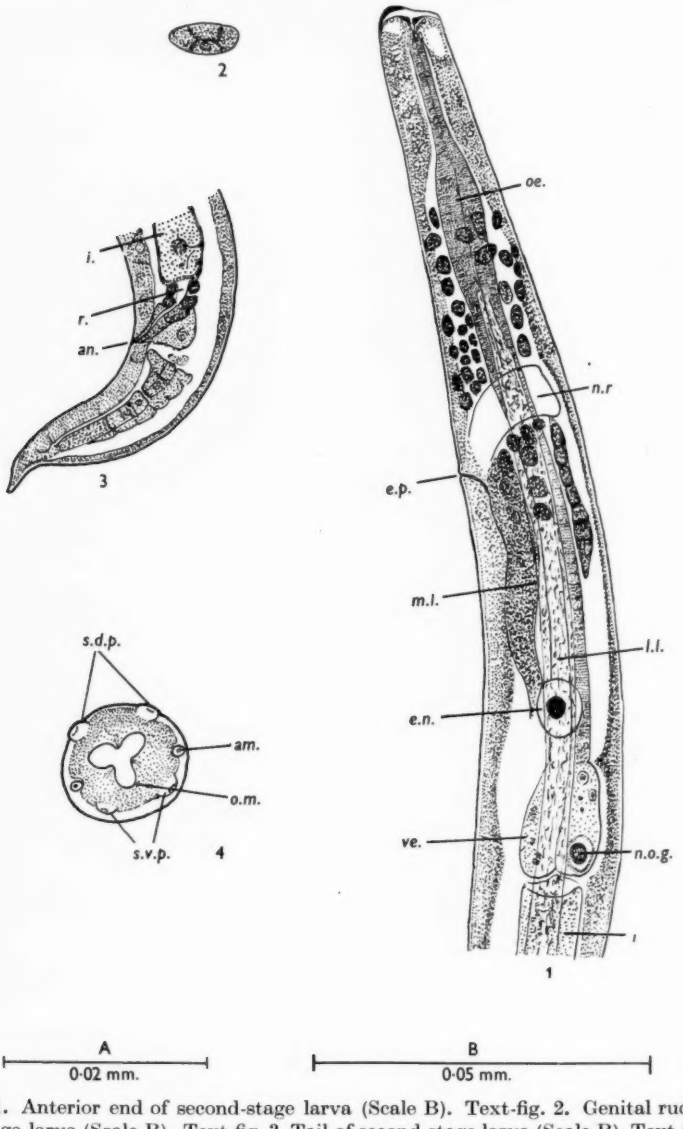
Experimental feeding to dogs and foxes of mice which had swallowed the embryonated eggs and harboured the second-stage larvae in their tissues, indicated in some instances that development of these larvae proceeded in the stomach and intestine. Few larvae were found in the somatic tissues of dogs, but at 3 days after infection second-stage larvae were found in the stomach wall; at 9 and 10 days after infection fourth-stage larvae were found in the intestine. In foxes fourth-stage larvae and adults were found in the intestine at 17 and 21 days after infection; larvae were also recovered from the somatic tissues, especially the lungs. Attempts to infect dogs and foxes with *T. cati* by feeding mice harbouring the larvae of *T. cati* in the tissues were unsuccessful.

V. DEVELOPMENT OF LARVAE

(*a*) *The second-stage larva* (Pl. IX, fig. 4)

Nichols (1956) investigated the morphology of living and heat-killed second-stage larvae which had been expressed from eggs and digested from mouse tissues. He found the larvae expressed from the egg to be morphologically identical with larvae recovered from mouse tissues except for slight growth of the excretory cell.

In the present series of observations second-stage larvae were recovered from the kidneys of a naturally infected dog, from the liver, lungs, kidneys, brain, heart



Text-fig. 1. Anterior end of second-stage larva (Scale B). Text-fig. 2. Genital rudiment of second-stage larva (Scale B). Text-fig. 3. Tail of second-stage larva (Scale B). Text-fig. 4. *En face* view of second-stage larva (Scale A).

muscle and alimentary tract of dogs experimentally infected by feeding embryonated eggs, and from the liver, lungs and alimentary tract of dogs and foxes infected by feeding mice. They were not found after birth in prenatally infected

puppies, and this indicated that the third stage is reached before birth. The larvae described below were fixed in hot 3% formalin, transferred to 70% alcohol containing 5% glycerin, gradually evaporated, and finally mounted in pure glycerin. Larvae expressed from eggs resembled those described by Nichols in every detail. Second-stage larvae collected from dog tissues differed from larvae expressed from eggs in that the cuticular thickening on the ventral lips was less prominent, so that the anterior end had a more truncate appearance. They differed also in that there was a more clearly differentiated ventriculus at the posterior end of the oesophagus, the lateral columns of the excretory cell extended to the posterior end, and the intestine in its anterior part appeared to consist of two rows of elongate cells enclosing the lumen of the intestine.

On the anterior end the second-stage larva bears a pair of subdorsal papillae, a pair of subventral papillae and a pair of amphids in the lateral position (Text-fig. 4). The mouth is directed slightly dorsad and the lips are not well defined in lateral view, though the margin of the subventral lips is strongly cuticularized (Text-fig. 1). The mouth is shaped like a clover leaf and is bounded by three triangular lips (Text-fig. 4). It opens into a strongly cuticularized vestibule, which enters the oesophagus. In its anterior part, for a distance of about 0·02 mm., the oesophagus is narrow, it then widens into a well-defined bulb which is surrounded by nuclei, it narrows again before it reaches the nerve ring. Behind the nerve ring it is obscured and pushed dorsally by the median lobe of the excretory cell, then widens again slightly before reaching the ventriculus (Text-fig. 1). The ventriculus is an elongate granular structure with a conspicuous nucleus which lies in the posterior part usually on the dorsal side. At its junction with the ventriculus, the oesophagus is not constricted. The rectum is funnel-shaped in lateral view and is lined by cuticle, the wall is composed of two elongate cells and several oval cells (Text-fig. 3). The genital rudiment (Text-fig. 2) is an elliptical structure lying ventrally to the intestine and about midway along its length.

No structural changes appeared to occur throughout the second-stage, except that the internal structure became more clearly defined, and some of the larvae showed a loosening of the cuticle indicating the onset of the second moult. The length of the second-stage larvae expressed from eggs and cleared in glycerin ranged from 0·39 to 0·44 mm., while those collected from dog tissues ranged from 0·34 to 0·44 mm. The second moult (Pl. IX, fig. 1) was observed in larvae from the lungs, heart and liver of egg-infected dogs (D68, D1, D2), and in the lungs and stomach wall of a mouse-infected fox (F1). The length of these moulting larvae was 0·37–0·45 mm. Other body measurements of second-stage larvae are shown in Table 3.

(*b*) *The third-stage larva* (Pl. IX, fig. 5)

Third-stage larvae were found in the liver, heart, lungs and stomach of puppies in the first week of life; in the liver, lungs and alimentary tract of puppies infected with eggs, and in the alimentary tract of dogs and foxes fed with infected mice. The anterior end (Text-fig. 5) resembles that of the second stage, although the lips appear more shallow, the oral aperture is triangular, and a pair of ventrolateral papillae are visible near the amphids (Text-fig. 9). Three triangular thicken-

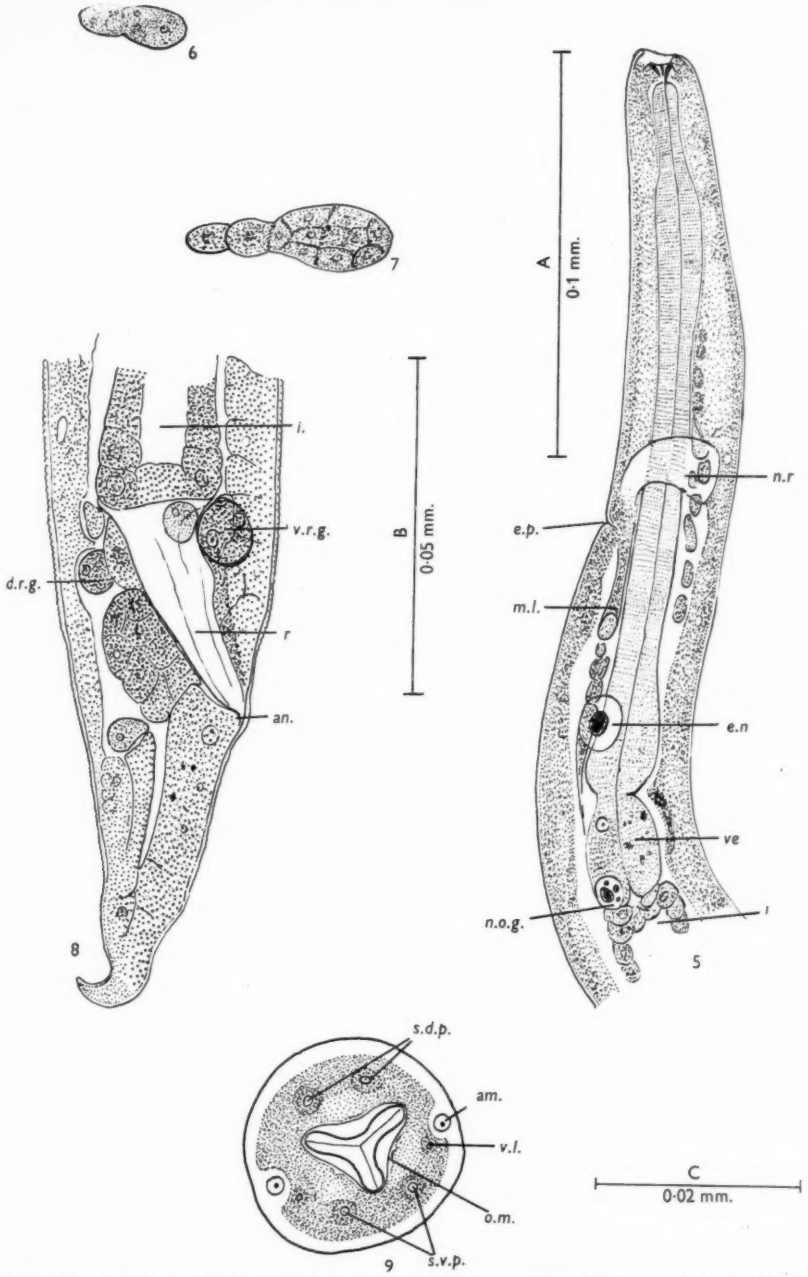
ings of the cuticle are conspicuous at the oral aperture, they are continuous with the lining of the oesophagus (Text-fig. 5). The oesophagus widens a short distance behind the mouth and maintains an even diameter until it meets the ventriculus; at this point it becomes slightly bulbous before being sharply constricted at the junction (Text-fig. 5). The ventriculus is a barrel-shaped granular structure with a conspicuous nucleus; in small third-stage larvae the nucleus was usually in its dorsal portion, but as the larva grows the nucleus appears to move into the ventral portion (Text-fig. 5). The excretory nucleus (Text-fig. 5) contains a

Table 3. *Measurements (mm.) of larval stages of* Toxocara canis

Stage	Length	Width	Excretory pore	Oesophagus	Ventriculus	Tail	Genital rudiment from anterior end
2nd (egg)	0·389	0·019	0·074	0·121	0·015	0·039	0·253
2nd (lung)	0·328	0·018	0·063	0·082	0·016	0·031	0·172
2nd (lung)	0·365	0·021	0·071	0·114	0·018	0·033	0·218
2nd (liver)	0·405	0·020	0·072	0·120	0·017	0·038	0·242
2nd (stomach)	0·445	0·024	0·077	0·117	0·022	0·032	0·277
3rd (stomach)	0·521	0·023	0·086	0·134	0·022	0·041	0·315
3rd (stomach)	0·546	0·029	0·084	0·136	0·021	0·039	0·323
3rd (stomach)	0·622	0·028	0·088	0·136	0·025	0·045	0·361
3rd (lung)	0·742	0·033	0·099	0·166	0·029	0·049	0·449
3rd (lung)	0·934	0·041	0·113	0·165	0·027	0·051	0·508
4th male	1·38	0·044	0·116	0·237	0·035	0·053	0·900
4th male	2·65	0·087	0·177	0·332	0·042	0·066	1·78
4th male	3·68	0·113	0·191	0·470	0·049	0·084	—
4th female	1·64	0·050	0·130	0·252	0·037	0·066	0·840
4th female	2·05	0·054	0·145	0·273	0·034	0·067	0·912
4th female	3·60	0·075	0·183	0·453	0·052	0·094	1·36 vulva
4th female	4·30	0·095	0·177	0·470	0·054	0·096	1·47 vulva
4th female	5·14	0·123	0·200	0·541	0·061	0·084	1·90 vulva

conspicuous, refractile disk and lies on the left side of the posterior third of the oesophagus; the median lobe of the excretory cell appears to be relatively smaller than in the second stage, the lateral columns extend almost the whole length of the larva. The intestine is a tube of even diameter lined by cuboidal or rectangular cells, the lumen becomes wider as the larva grows. The rectum is funnel-shaped and in its dorsal wall is a bunch of cells which may represent, in male larvae, the primordium of the spicules and spicule sheaths. Situated dorsal and ventral to the rectum are several well-defined cells (Text-fig. 8); they probably become the rectal glands which are conspicuous in the fourth and adult stages. The tip of the tail is curved dorsally and the degree of curvature appeared to be some indication of the age of the larva. The body cavity is clearly defined in all regions behind the nerve ring, it contains the genital rudiment on the ventral side about mid-way along the length of the intestine. In small third-stage larvae the genital rudiment is oval or elliptical in outline (Text-fig. 6), but in more developed larvae an anterior prolongation appears (Text-fig. 7).

The smallest third-stage larva found was 0·46 mm. in length, the largest was 1·26 mm. Other measurements of third-stage larvae are shown in Table 3. The



Text-fig. 5. Anterior end of third-stage larva (Scale A). Text-fig. 6. Genital rudiment of third-stage larva (length 0·6 mm.) (Scale B). Text-fig. 7. Genital rudiment of third-stage larva (length 0·9 mm.) (Scale B). Text-fig. 8. Tail of third-stage larva (Scale B). Text-fig. 9. *En face* view of third-stage larva (Scale C).

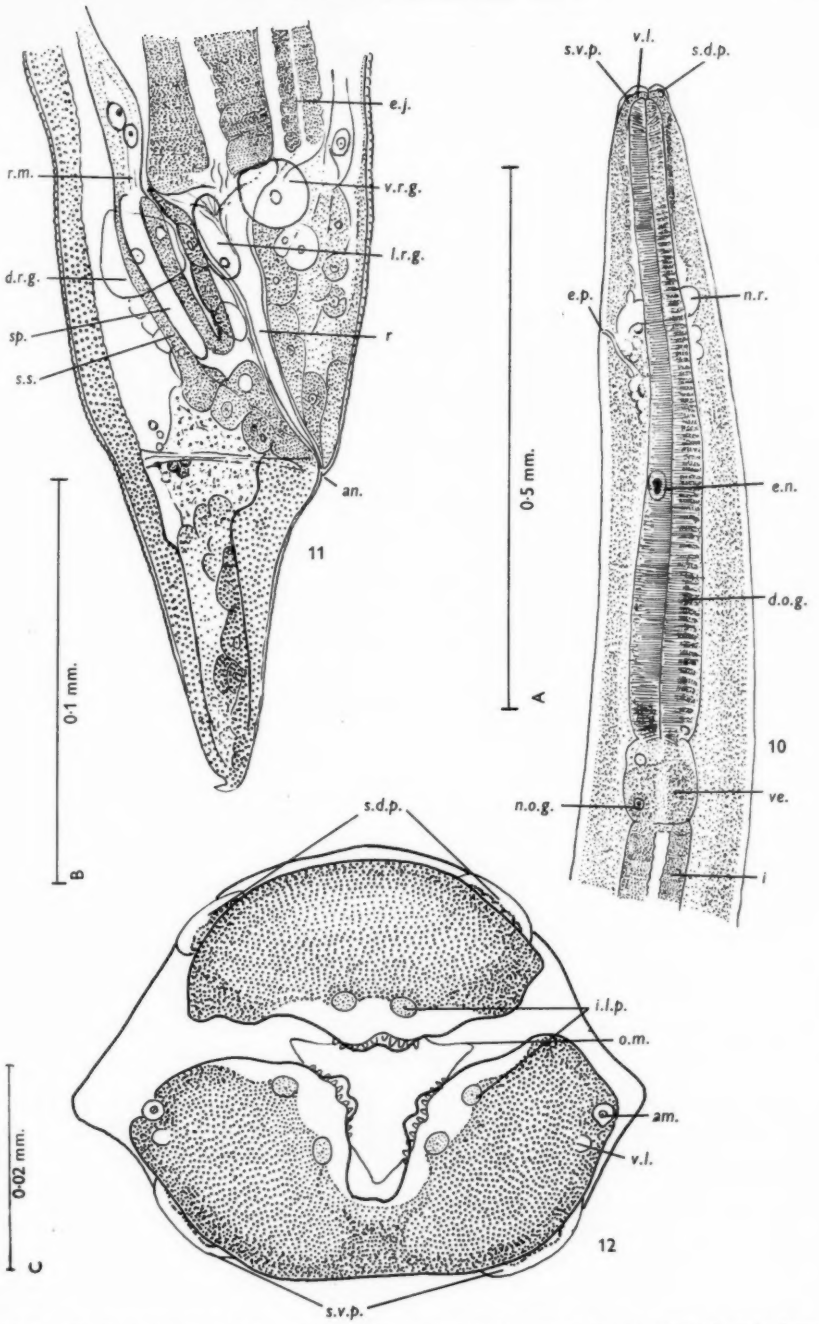
third moult (Pl. IX, fig. 2) was observed in the lungs of a naturally infected week-old puppy (D73), in the lungs and alimentary tract of egg-infected puppies (D2, D76, D77) and in the stomach contents of a fox (F2) infected by feeding mice. The lengths of these moulting larvae ranged between 0·98 and 1·3 mm.

(*c*) *The fourth-stage larva* (Pl. IX, fig. 6)

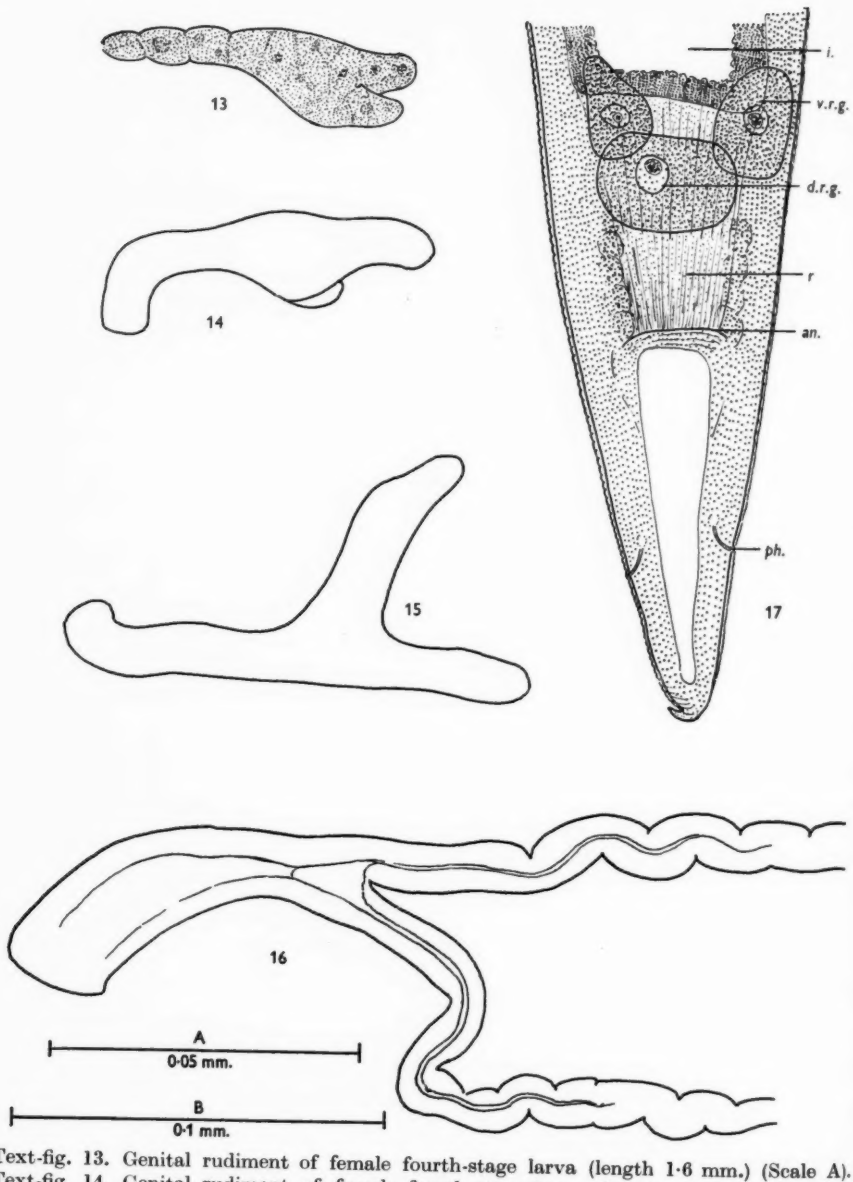
In naturally infected puppies fourth-stage larvae began to appear in the intestine 3 days after birth (D44). In young pups infected with eggs, fourth-stage larvae were found in the alimentary tract at 9 days (D2, D77), 16 days (D3) and 23 days (D4) after infection. In dogs infected with mice, fourth-stage larvae were found in the intestine of a 5-week-old puppy 10 days after infection (D14), and in a 7-week-old puppy 9 days after infection (D75). In foxes infected with mice, fourth-stage larvae were found in the alimentary tract at 17 (F1) and 21 (F2) days after infection.

The fourth-stage larva differs from the third-stage in the presence of three rounded lips (Text-fig. 12). The subventral lips each have a single oval subventral papilla, a smaller lateral papilla, and an amphidial pore. The dorsal lip has two large papillae. There are also six oval areas near the inner border of the lips which probably represent an internal ring of labial papillae. Short dentigerous ridges are visible on the external margin of each lip. The oesophagus is distinctly striated by the follicles of the dorsal oesophageal gland (Text-fig. 10). The diameter of the oesophagus increases gradually as it extends posteriorly; at its junction with the ventriculus there is no marked constriction. The ventriculus is a granular structure without striations and is clearly separated from the oesophagus and intestine, it contains a conspicuous nucleus in the posterior part of the ventral section. The dorsal oesophageal gland extends almost the whole length of the oesophagus, the follicles becoming no longer visible about mid-way between the nerve ring and the anterior end. The subventral glands were not distinct. The excretory nucleus is not as conspicuous as it is in the third stage, it contains several refractile particles and lies alongside the posterior part of the oesophagus in the left side (Text-fig. 10). The intestine has a wide lumen and is lined by columnar cells. The rectum is lined with cuticle and in lateral view it is funnel-shaped (Text-fig. 11), but in ventral view it is rectangular; it is associated with three conspicuous rectal glands in the female, one large dorsal and two smaller ventrals (Text-fig. 17). In male larvae there appear to be two additional glands in a lateral position (Text-fig. 11). The body cavity is capacious, in the tail region it is filled with cells.

The genital rudiment undergoes considerable development during the fourth stage. Soon after the third moult is completed it is possible to differentiate male and female larvae by the development of the genital rudiment. In male larvae it elongates in a posterior direction forming a filamentar projection running backwards below the intestine. At a length of 2·7 mm. the primordia of the spicules and ejaculatory duct were visible, and at a length of 3·7 mm. the developing spicules and rectractor muscles were already visible in the dorsal wall of the rectum (Text-fig. 11). In female larvae the genital rudiment elongates in both anterior and posterior directions. Anteriorly, a single projection is formed



Text-fig. 10. Anterior end of fourth-stage larva (Scale A). Text-fig. 11. Tail of male fourth-stage larva (Scale B). Text-fig. 12. *En face* view of fourth-stage larva (Scale C).



Text-fig. 13. Genital rudiment of female fourth-stage larva (length 1·6 mm.) (Scale A).
Text-fig. 14. Genital rudiment of female fourth-stage larva (length 2 mm.) (Scale A).
Text-fig. 15. Genital rudiment of female fourth-stage larva (length 2·6 mm.) (Scale A).
Text-fig. 16. Genital rudiment of female fourth-stage larva (length 3·5 mm.) (Scale A).
Text-fig. 17. Tail of female fourth-stage larva (Scale B).

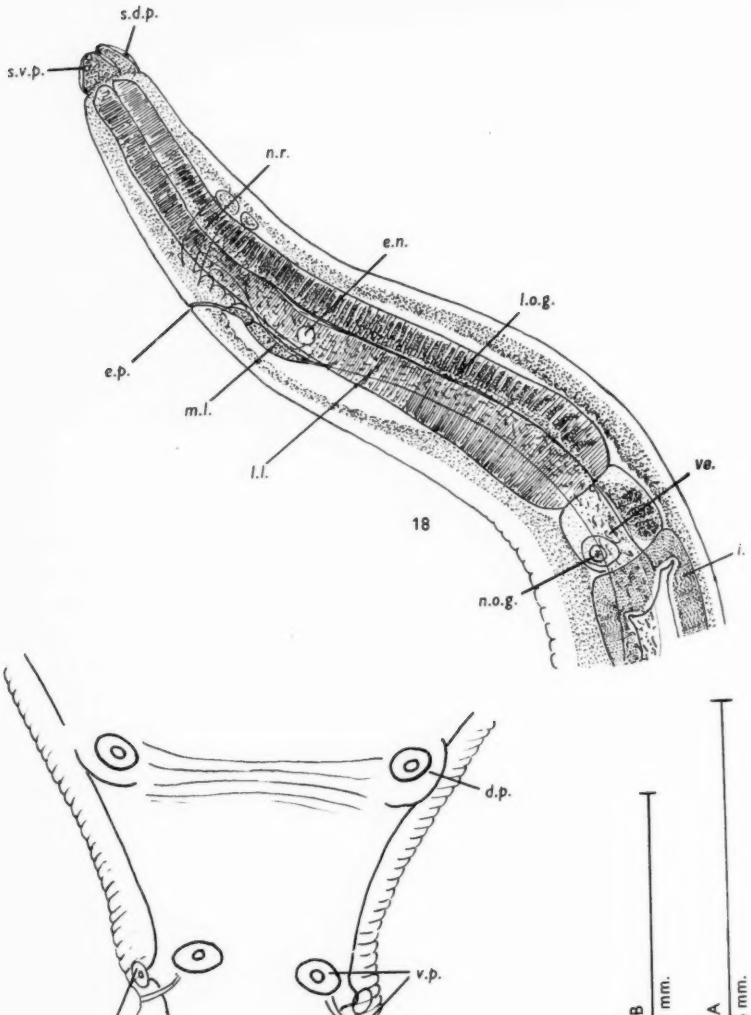
(Text-fig. 13) which curves ventrally and constitutes the undivided part of the uterus and the vagina (Text-fig. 14). The posterior prolongation, when the larvae are about 2 mm. long, divides into two, one continues growing as a filament along the left side of the intestine, the other crosses to the right side below the intestine and progresses in a similar manner (Text-fig. 15); these posterior prolongations become the two uteri and ovaries. At a length of 3 mm. the female organs appear like a wishbone, the undivided portion of the uterus and vagina appear as a curved tube, the latter with a definite lumen. At 4 mm. the female organs appear as a tuning-fork extending along the intestine as undulating filaments (Text-fig. 16) and the phasmids become visible on each side of the tail. These consist of a depression in the cuticle to which runs a minute canal (Text-fig. 17).

The smallest fourth-stage found measured 1·2 mm. in length, the largest measured 6·3 mm. in length. Other measurements of fourth-stage larvae are shown in Table 3. The fourth moult (Pl. IX, fig. 3) was observed in the intestinal contents of egg-infected and mouse-infected dogs. The lengths of these moulting larvae was 5·4–7·4 mm.

The adult stage (Pl. IX, fig. 7)

Adults were found only in the intestinal contents. They were easily distinguished from fourth-stage larvae by the segmentation of the cuticle. At the time of the fourth moult the cuticular rings in the posterior third of the body are about five times as wide as those of the adult stage lying within the sheath. At the completion of the fourth moult, the lips assume the adult characteristics, with three prolongations of the pulp into each lip and a constriction between the body and posterior border of each lip. The labial papillae are the same in arrangement, but more conspicuous than those in the fourth stage. The nucleus of the dorsal oesophageal gland lies in the posterior part of the ventral portion of the ventriculus (Text-fig. 18); the nuclei of the subventral glands lie in the anterior part of the ventral portion, they are smaller than the dorsal nucleus. In the female the vulva becomes apparent as an elongate slit on the ventral slide and before the fourth moult the phasmids become visible. These are minute projections of the parenchyma on each side of the tail, each containing a canal which runs to a slight depression of the surface. After the fourth moult there appear on each side of the tail of the male, two lateral postcloacal papillae, two ventro-lateral post-cloacal papillae and a phasmid (Text-fig. 19). A double papilla appears on each side of the cloaca, and 19–24 precloacal papillae are also visible.

As the adult grows in length, certain other changes become evident, the dentigerous ridges become more extensive, the lateral alae, which extend the whole length of all larval stages and young adults, become restricted to the anterior part of the body and finally disappear at a length of 20–30 mm. The cervical alae are evident at 13 mm., they merge into the lateral alae in the region of the posterior third of the oesophagus. The smallest male adult observed measured 5·8 mm. in length, the smallest female 6·2 mm., other body measurements are shown in Table 4.



Text-fig. 18. Anterior end of adult (Scale A). **Text-fig. 19.** Tail of adult male (Scale B).

Table 4. *Measurements (mm.) of adult stages of* Toxocara canis

Stage	Length	Width	Excretory pore	Oesophagus	Ventriculus	Tail	Spicules	Vulva from ant. end
Adult male	5·8	0·170	0·250	0·623	0·074	0·096	0·205	—
Adult male	9·9	0·243	0·300	0·864	0·125	0·115	0·285	—
Adult male	13	0·294	0·324	1·06	0·115	0·098	0·313	—
Adult male	15	0·320	0·360	1·19	0·160	0·154	0·432	—
Adult male	28	0·492	0·528	1·85	0·198	0·160	0·627	—
Adult female	6·9	0·192	0·246	0·660	0·096	0·131	—	2·6
Adult female	9·4	0·186	0·271	0·792	0·102	0·150	—	3·1
Adult female	15	0·301	0·320	1·13	0·134	0·208	—	4·2
Adult female	28	0·444	0·504	1·70	0·192	0·300	—	7·7
Adult female	35	0·492	0·504	2·01	0·246	0·480	—	9·2
Adult female	45	0·660	0·600	2·48	0·288	0·470	—	13·0

VI. DISCUSSION

Comparison of the stages of development of the larvae of *T. canis* and *T. cati*, indicates that the morphological features of their developmental stages are almost identical (cf. Sprent, 1956). On the other hand, the migratory behaviour of the larvae show certain striking differences. Sprent (1955) reported that a relatively large proportion of *T. canis* larvae migrating in the tissues of mice were found to be distributed to the brain, while the larvae of *T. cati* were only rarely recovered from the brain. This has been confirmed in later experiments [cf. Table 2 in the present paper with Table 2 (Sprent, 1956)]. It was found that the width of second-stage *T. canis* was slightly greater than that of *T. cati* larvae, and it was suggested that the difference in behaviour might be related to the difference in width in relation to the blood vessels of the host (Sprent, 1955).

It is evident that a difference is also manifested by the larvae of these two species in their distribution to foetal tissues. For, while prenatal infection is evidently the usual mode of infection with *T. canis* in dogs, it is evidently rare in the case of *T. cati*. It was shown (Sprent, 1956) that when *T. cati* eggs containing second-stage larvae were swallowed by cats, the larvae migrated through the intestinal wall, liver and lungs. Although a proportion of these larvae was distributed to the somatic tissues, e.g. muscles, no evidence was found to indicate that prenatal infection occurred. Many reached the alimentary tract and underwent further development, but it was not established that they reached this site by tracheal migration. The present investigation showed that the behaviour of *T. canis* larvae, in the same circumstances, differed in that the larvae were evidently unable to reach the alimentary tract of dogs, except in very young animals (birth to 3 weeks), but migrated in relatively large numbers to the somatic tissues. In other words, it was evident that the migratory behaviour of the larvae of each of these two species predisposes to different modes of infection. The behaviour of the larvae of *T. canis*, resulting as it does in the deposition of second-stage larvae in the somatic tissues, predisposes to prenatal infection or infection by cannibalism, and except in very young animals precludes direct infection by eggs. In contrast, the behaviour of

T. cati larvae in cats predisposes to direct infection with eggs, but evidently precludes prenatal infection.

When ingestion of infected mice was used as the method of infection, a much lower degree of infection resulted in the dog when fed with mouse tissues harbouring second-stage larvae of *T. canis*, than was found to occur in the cat infected in the same way with *T. cati* (Sprent, 1956). In cats, infection by this means was found to be more intensive and quicker to develop than infection by eggs, perhaps indicating that infection through an intermediate host is the usual method.

There are many aspects of the life history of these two parasites which await further elucidation, especially those relating to the precise mechanism of prenatal infection. Nevertheless, it is probable that natural infection may occur in several different ways, e.g. by cannibalism, by carrion-eating or predation, by ingestion of food contaminated with eggs, and by intra-uterine migration. As a result of adaptations to prevailing modes of nutrition in a particular species of host, certain differences have evolved in the migratory behaviour of the larvae, which predispose to certain modes of infection. It must be emphasized that these two parasites occur in a relatively wide range of hosts (the hosts of *T. canis* are shown in Table 1, those of *T. cati* have been previously listed (Sprent, 1956)). Hence it is necessary that the larvae should be able to adapt themselves to any of the above methods of infection, while retaining the ability to change their behaviour if they gain access to a host of a different species. Thus, the usual mode of infection for *T. canis* in the dog, i.e. the migration of ingested second-stage larvae into the liver of the foetus, is possibly an adaptation to the non-predatory habits of the dog. On the other hand, different adaptations have probably occurred in the case of wild foxes, jackals, wolves, dingoes and other Canidae, whereby infection occurs through predation, cannibalism, carrion-eating, or other nutritional habits.

VII. SUMMARY

1. Evidence from previous literature shows that puppies are commonly infected with larvae of *Toxocara canis* at birth and that prenatal infection can be produced by feeding embryonated eggs to pregnant bitches.

2. Observations on fifty-eight dogs in Brisbane showed that all of twenty-nine puppies 1–6 months old were infected, while only three out of twenty-nine dogs over 6 months old were infected.

3. In naturally infected puppies, 1–3 weeks old, it was found that at birth third-stage larvae were present in the lungs. Third-stage larvae continued to appear in the lungs for the first week of life; their length was 0·6–1·3 mm.

4. Third-stage larvae were found in the stomach on the day after birth; at 3 days after birth fourth-stage larvae were found in the intestine.

5. It appeared likely that the second moult occurs in puppies before birth, and that the third moult takes place at a length of 1·0–1·3 mm. in the lungs and stomach within the first week of life.

6. By the beginning of the second week, fourth-stage larvae were fully grown and had commenced the fourth moult at a length of 5–7 mm.

7. Throughout the second and third weeks, adults grew rapidly, reaching a length of about 67 mm. by the end of the third week, but no eggs had appeared in the faeces at this time.

8. Experimental infection of mice with eggs showed that the larvae were distributed to the somatic tissues, few reaching the alimentary tract. The larvae did not progress beyond the second stage in the tissues of mice.

9. Experimental infection of dogs with eggs showed that in dogs over 5 weeks old the larvae were distributed to the somatic tissues and did not reach the alimentary tract. The larvae did not progress beyond the second stage, though some of them showed signs of commencing the second moult. In contrast, 1 to 3-week-old puppies infected in the same way were found to harbour larvae in the alimentary tract. Though some of these larvae were probably derived from a naturally acquired prenatal infection, it was evident from the progress of development that the experimental infection resulted in the presence of second-stage larvae in the liver and lungs and that these larvae underwent the second moult, commenced development as third-stage larvae in the lungs, migrated into the stomach and developed to the adult stage in the intestine.

10. Experimental infection of dogs and foxes with mice harbouring second-stage larvae in the tissues showed that, in some instances, development of larvae proceeded in the alimentary tract. No evidence of somatic migration was found in dogs infected in this way, but in foxes second-stage larvae were found in the lungs.

11. The structure and development of the second, third and fourth stage is described in detail and found to resemble closely the development of *T. cati* larvae (Sprent, 1956). Second-stage larvae from dog tissues had a length of 0·34–0·44 mm. and the second moult occurred at a length of 0·37–0·44 mm. Third-stage larvae varied in length from 0·46 to 1·36 mm., and the third moult occurred at a length of 0·98–1·3 mm. Fourth-stage larvae measured 1·2–6·3 mm., though moulting fourth-stage larvae were observed up to a length of 7·4 mm. Sexual differentiation occurred during the fourth stage and was evident at a length of about 1·5 mm.

12. The migratory behaviour of the larvae of *T. canis* and *T. cati* is compared and discussed in relation to their wide range of hosts. It is concluded that differences in migratory behaviour are adaptations to prevailing modes of nutrition and it is suggested that the somatic migration occurring in dogs is an adaptation to the non-predatory habits of this particular host.

The writer's thanks are due to his wife for rearing many of the puppies used in these experiments, to Mr S. C. Ellis and Mr J. J. Mines for their able technical assistance, and to Dr J. C. Pearson for his interest and help. The writer also wishes to acknowledge the help in obtaining certain references that he has received from Professor R. T. Leiper, F.R.S., Director of the Commonwealth Bureau of Agricultural Parasitology, St Albans, England, and from Mr Allen McIntosh of the Agricultural Research Service of the United States Department of Agriculture. The work was supported by a research grant from the University of Queensland. The photographs were taken by Mr E. Hollywood of the Department of Photography, University of Queensland.

REFERENCES

AUGUSTINE, D. L. (1927). Development in prenatal infestation of *Belascaris*. *J. Parasit.* **13**, 256–9.

BAYLIS, H. A. & DAUBNEY, R. (1922). Report on the parasitic nematodes in the collection of the Zoological Survey of India. *Mem. Indian Mus.* **7**, 263–347.

BEAVER, P. C., SNYDER, C. H., CARRERA, G. M., DENT, J. H. & LAFFERTY, J. W. (1952). Chronic eosinophilia due to *visceral larva migrans*. *Pediatrics, Springfield*, **9**, 7–19.

CANAVAN, W. P. N. (1929). Nematode parasites of vertebrates in the Philadelphia Zoological Garden and vicinity. I. *Parasitology*, **21**, 63–102.

CANAVAN, W. P. N. (1931). Nematode parasites of vertebrates in the Philadelphia Zoological Garden and vicinity. II. *Parasitology*, **23**, 196–229.

COWAN, I. M. (1947). The timber wolf in the Rocky mountain national parks of Canada. *Canad. J. Res.* D, **25**, 139–74.

DAVAINE, C. J. (1863). Sur la constitution de l'œuf de certains entozoaires et sur la propriété de se développer à sec. *C.R. Soc. Biol., Paris*, **4**, 273–8.

FÜLLEBORN, F. (1921). Askarisinfektion durch Verzehren eingekapselter Larven und über gelungene intrauterine Askarisinfektion. (Vorläufige Mitteilung.) *Arch. Schiffs.- u. TropenHyg.* **25**, 367–75.

HADLEY, F. B., WARWICK, B. L. & GILDOW, E. M. (1924). Prenatal infestation of fox pups with belascardis. *J. Amer. Vet. Med. Ass.* **19**, 301–7.

HERING, E. A. VON (1873). Beiträge zur Entwicklungsgeschichte einiger Eingeweide-würmer. *Jh. Ver. vaterl. Naturk. Württemb.* **29**, 305–67.

HITCHCOCK, D. J. (1953). Incidence of gastro-intestinal parasites in some Michigan kittens. *N. Amer. Vet.* **34**, 428–9.

HOEPLI, R., FENG, L. C. & LI, F. (1949). Histological reactions in the liver of mice due to larvae of different Ascaris species. *Peking Nat. Hist. Bull.* **18**, 119–32.

KREIS, H. A. (1952). Helminthologische Untersuchungen in schweizerischen Tierpärken und bei Haustieren. *Schweiz. Archiv Tierheilk.* **94**, 499–583.

LEIPER, R. T. (1907). Two new genera of nematodes occasionally parasitic in man. *Brit. Med. J*, **1**, 1296–8.

MATOFF, K. (1949). [*Experimental Investigations on the Migration of* Toxocara canis, Toxocaris leonina *and* Neoascaris vitulorum], Year book of the Rural Academy, Sofia. (In Russian.) XXV, 593–659.

MOZGOVI, A. A. (1953). [*Ascaridata of Animals and Man and Diseases Caused by Them.*] 2 vols. (In Russian.) U.S.S.R.: Leningrad, Academy of Sciences.

NELSON, H. (1852). On the reproduction of the *Ascaris mystax*. *Phil Trans.* pp. 563–94.

NICHOLS, R. L. (1956). The etiology of *visceral larva migrans*. I. Diagnostic morphology of infective second-stage *Toxocara* larvae. *J. Parasit.* **42**, 349–62.

NIFONTOV, S. N. (1949). Importance of intra-uterine invasion in the epizoology of *Toxocara* infection in dogs. *Veterinariia, Moscow*, **26**, 32–4.

PETROV, A. M. (1930) The work of the 61st Union Helminthological Expedition on Sakhalin Island. [Russian text—original not seen.] *Trudy Gosudarstv. Inst. Eksper. Vet.* **6**, 110–26.

PETROV, A. M. (1941). (Importance de l'invasion intrauterine dans l'epizootologie de la toxocarose des renards noirs-argentés.) [Russian text: French Summary.] *Vestn. selkhoz. Nauk. Vet.* **3**, 84–92.

RAILLIET, A. & HENRY, A. C. (1911). Recherches sur les ascarides des carnivores. *C.R. Soc. Biol. Paris*, **70**, 12–15.

SCHMID, F. (1934). Über Parasiten und parasitäre Veränderungen der Harnorgane bei Silberfüchsen. *Berl. tierärtzl. Wschr.* **50**, 33–6.

SHILLINGER, J. E. & CRAM, E. B. (1923). Parasitic infestation of dogs before birth. *J. Amer. Vet. Med. Ass.* **63**, 200–3.

SHOHO, C. (1955). *Helminthic diseases of the central nervous system and intrauterine foetal infection by helminths.* (*Significance of histologic diagnosis in somatic Helminthiasis.*) *Rev. iber. Parasit.* (Tomo Extraordinario), pp. 927–51.

SMITH, L. F. (1943). Internal parasites of the red fox in Iowa. *J. Wildlife Mgmt*, **7**, 174–8.

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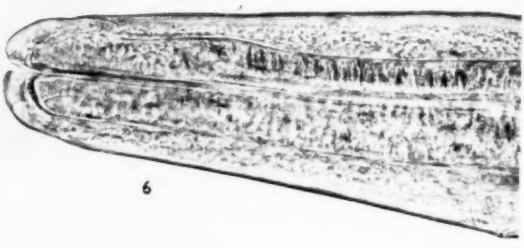

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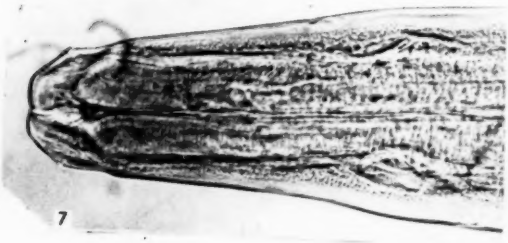

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(*Facing p.* 209)

SPRENT, J. F. A. (1952). On the migratory behaviour of the larvae of various Ascaris species in white mice. I. Distribution of larvae in tissues. *J. Infect. Dis.* **90**, 165–76.

SPRENT, J. F. A. (1953). On the migratory behaviour of the larvae of various Ascaris species in white mice. II. Longevity of encapsulated larvae and their resistance to freezing and putrefaction. *J. Infect. Dis.* **92**, 114–17.

SPRENT, J. F. A. (1955). On the invasion of the central nervous system by Nematodes. II. Invasion of the nervous system in Ascariasis. *Parasitology*, **45**, 41–55.

SPRENT, J. F. A. (1956). The life history and development of *Toxocara cati* (Schrank 1788), in the domestic cat. *Parasitology*, **46**, 54–78.

TINER, J. D. (1953). The migration, distribution in the brain, and growth of ascarid larvae in rodents. *J. Infect. Dis.* **92**, 105–13.

VEVERS, G. M. (1923). On the parasitic nematoda collected from mammalian hosts which died in the Gardens of the Zoological Society of London during the years 1919–1921; with a description of three new genera and three new species. *Proc. zool. Soc. Lond.* (1922), pt. 4, 901–19.

YAMAGUTI, S. (1941). Studies on the helminth fauna of Japan. Part 35. Mammalial nematodes, II. *Jap. J. Zool.* **9**, 409–39.

YUTUC, L. M. (1949). Prenatal infection of dogs with ascarids, *Toxocara canis* and hookworms, *Ancylostoma caninum*. *J. Parasit.* **35**, 358–60.

LIST OF ABBREVIATIONS

am.	amphid	*n.o.g.*	nucleus of oesophageal gland
an.	anus	*o.e.*	oesophagus
d.o.g.	dorsal oesophageal gland	*o.m.*	oral margin
d.p.	double post-anal papilla	*ph.*	phasmid
d.r.g.	dorsal rectal gland	*r.*	rectum
e.j.	ejaculatory duct	*r.m.*	rectractor muscle
e.n.	excretory nucleus	*s.d.p.*	subdorsal papilla
e.p.	excretory pore	*s.v.p.*	subventral papilla
i.	intestine	*sp.*	spicule
i.l.p.	internal labial papilla	*s.s.*	spicule sheath
l.l.	lateral excretory lobe	*v.e.*	ventriculus
l.p.	lateral post-anal papilla	*v.l.*	ventro-lateral papilla
l.r.g.	lateral rectal gland	*v.p.*	ventral post-anal papilla
m.l.	median excretory lobe	*v.r.g.*	ventral rectal gland
n.r.	nerve ring		

EXPLANATION OF PLATE IX

Fig. 1. Second moult from lung of fox. × 300.
Fig. 2. Third moult. Stomach of puppy. × 150.
Fig. 3. The fourth moult, posterior end. × 300.
Fig. 4. Anterior end of second-stage larva. × 500.
Fig. 5. Anterior end of third-stage larva showing lips. × 500.
Fig. 6. Anterior end of fourth-stage larva. × 500.
Fig. 7. Anterior end of adult. × 500.

(*MS. received for publication* 3. VIII. 1957.—Ed.)

AN ANALYSIS OF THE DEVELOPMENT OF RESISTANCE TO METACHLORIDINE IN CLONES OF *PLASMODIUM GALLINACEUM*

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CONTENTS

		PAGE
I.	Introduction	210
II.	Methods	211
III.	The character of infections produced by single parasites of *Plasmodium gallinaceum*	213
IV.	The effect of a single course or of two courses of treatment with metachloridine upon *Plasmodium gallinaceum*	213
V.	The effect of serial treatment with 0·1 mg./20 g. doses of metachloridine upon the development of resistance to the drug in clone A (strain I)	215
VI.	The effect of serial treatment with 0·05 mg./20 g. doses of metachloridine upon the development of resistance to the drug in clone A (strain II)	217
VII.	The effect of serial treatment with 0·25 mg./20 g. doses of metachloridine upon the development of resistance to the drug in clone A (strain III)	218
VIII.	A comparison of the development of resistance to metachloridine in strains of clone A treated with 0·05 mg./20 g. (strain IV) and 0·25 mg./20 g. doses of metachloridine (strain V) and with 0·05 mg./20 g. (strain VI) and 1·0 mg./20 g. doses (strain VII)	219
IX.	The effect of variations in the size of the inoculum upon the rate of development of resistance to metachloridine in clone A (strains VIII, IX, X, XI and XII)	221
X.	The effect of serial courses of treatment with 0·1 mg./20 g. of metachloridine upon *Plasmodium gallinaceum* clone B (strain XIII)	223
XI.	The effect of the size of the inoculum upon the rate of development of resistance to metachloridine in *Plasmodium gallinaceum* clone B (strains XIV and XV)	224
XII.	The effect of inocula consisting of mixtures of metachloridine-resistant and normal parasites	225
XIII.	Discussion	228
XIV.	Summary	233
	References	233

I. INTRODUCTION

The problem of the mechanism by which resistance develops has been studied extensively in bacteria, their short generation time and ability to grow in culture media making them particularly suitable for this work. Since it is not possible to maintain malaria parasites in culture, drug resistance in these organisms must be studied in strains of plasmodia maintained in laboratory animals. The conditions of the experiment, therefore, are less uniform and less under the control of the worker than in experiments upon bacteria, since such factors as variations in the innate and acquired immunity of the hosts with their consequent effects upon

parasite numbers, and differences in rate of absorption and blood levels of the drug cannot be eliminated. In addition, the number of experiments which it is practicable to carry out and the number of strains which can be maintained, are fewer than with bacteria.

Hitherto, no study of the development of resistance to antimalarial drugs has been carried out upon pure strains of plasmodia derived from single parasites. Moreover, with the exception of the work of Williamson & Lourie (1947) in which infections induced by inocula of known numbers of parasitized erythrocytes were treated with proguanil, no effort has been made to define the numbers of parasites exposed to the action of the drug. The main object of previous work has been to obtain strains of plasmodia resistant to various drugs and to study the pattern of cross-resistance rather than to analyse the conditions under which resistant strains arise.

The following account describes the production of resistance to metachloridine (3-metanilamido-5-chloropyrimidine) in clones* of *Plasmodium gallinaceum*, and records the results of experiments designed to ascertain whether the number of parasites exposed to the drug and the size of the dose affect the rate at which resistant infections become established. Metachloridine was chosen because it had previously been shown to produce resistance in *P. gallinaceum* relatively rapidly (Bishop & McConnachie, 1953). In the experiments to be described every effort was made to maintain the substrains under strictly comparable conditions with the exception of the variable being studied.

That infections could be produced by the inoculation of single malaria parasites had already been shown. Thus Coulston & Manwell (1941) succeeded in infecting a canary with a single schizont of *P. circumflexum*, and, later, Downs (1947) obtained infections in young chicks inoculated intracardially with single erythrocytes containing single parasites of *P. gallinaceum*. Previously, Demidowa (1934) had obtained infections in two birds inoculated with single erythrocytes infected with *P. praecox* and in one bird inoculated with an erythrocyte parasitized with *P. cathemerium*, but was not certain that each erythrocyte contained only one parasite.

II. METHODS

The strain of *P. gallinaceum* used in these experiments was the parent strain from which strains resistant to metachloridine were previously developed (Bishop & McConnachie, 1953). In attempting to induce infection by a single malaria parasite it is important that the erythrocyte isolated for injection should contain a very small trophozoite, since a large parasite might conceal a smaller one. Very light infections, in which the majority of parasitized erythrocytes contained only a single, very small trophozoite, were therefore used in the isolation experiments. The blood was diluted with a mixture of equal parts of citrate saline and normal chick serum until a small drop contained only a few corpuscles well separated from each other. A very small drop of the diluted parasitized blood was put upon a

* A clone has been defined by Sonneborn (1947) as consisting of the progeny of a single individual produced by successive fissions in the complete absence of all fertilization processes.

coverslip and small drops of the serum-saline mixture were also put on to the same coverslip at widely spaced and regular intervals. The corner of the coverslip nearest to the drop containing parasitized blood was marked with a minute spot of ink to prevent confusion when the coverslip was reversed after an erythrocyte containing a single parasite had been isolated in one of the drops. The coverslip was then inverted over a small chamber of the type used in micromanipulation, the walls of which had been smeared with vaseline to hold the coverslip in place. A Singer micromanipulator was used for manipulating the micropipette with which the isolation was performed. The isolation was carried out under a $\frac{1}{6}$ in. objective, but the final isolation was confirmed under a $\frac{1}{12}$ in. objective.

Erythrocytes were isolated into the drops of serum-saline until it was observed that a drop contained a single corpuscle containing one small trophozoite. The position of the drop was carefully noted with reference to the ink spot, and the coverslip was then reversed. The drop was drawn through a no. 18 stainless-steel hypodermic needle into a 1 ml. all-glass tuberculin syringe containing 0·2 ml. of the serum-saline. The content of the syringe was injected into the jugular vein of a 4-day-old chick.

In studying the development of resistance to metachloridine two clones, A and B, were used, the general procedure being as follows. A group of chicks, 45–60 g. in weight, was inoculated intravenously with parasitized blood from a bird infected with one of the clones. Three of the recipients, which served as untreated controls, each received an inoculum of approximately 5×10^7 parasites calculated from blood and parasite counts, the blood being mixed with citrate-saline solution to give the required dilution. An inoculum of this size produced a heavy infection in untreated birds by the 4th day following inoculation, 50–90 % of the erythrocytes being parasitized. The number of parasites inoculated into each of the remaining birds varied with the nature of the experiment, but each variable was tested upon a group of three or more birds, the usual number being four. Metachloridine, made soluble in water by the addition of a small volume of normal sodium hydroxide, was administered from a graduated syringe by a fine catheter tube into the stomach of the bird, the dose being calculated in mg. per 20 g. of body weight of bird. The drug was given morning and evening for $3\frac{1}{2}$ days, the first dose being given immediately after the birds had been inoculated. Such a series of doses will be referred to as a course of treatment.

On the 4th day following inoculation, a blood film was made from each bird, and the intensity of infection was assessed as the number of parasitized erythrocytes per 500 erythrocytes (p.e./500 e.). After a course of treatment with an inhibitory dose of drug the number of parasitized erythrocytes was small; it was therefore necessary for the parasites to multiply before the strain could be exposed to a further course of treatment. Since exo-erythrocytic parasites tend to increase in number as infections progress, and since it was desirable to avoid, as far as was possible, the complication of erythrocytic parasites derived from this source, the erythrocytic parasites which had survived a course of treatment were not allowed to multiply in the birds in which they had been exposed to drug treatment but were inoculated into untreated birds. 0·2–0·9 ml. of blood, the volume depending

upon the intensity of infection, was removed from each bird at the end of the course of treatment and mixed with heparin-saline solution in the proportion of 1 part of heparin-saline to 9 parts of blood, and injected into a clean bird, each recipient receiving blood from a different bird. When the parasites had multiplied sufficiently, groups of birds were inoculated from these untreated birds, each group being inoculated with the desired number of parasites from a different untreated bird. The strain was then subjected to a further course of drug treatment. Practical limitations made it impossible, unfortunately, to test the infections in each untreated bird for resistance, but in each experiment the infections which developed most rapidly in untreated birds, and were therefore presumably least damaged by the drug, were used as inocula in the next course of treatment.

III. THE CHARACTER OF INFECTIONS PRODUCED BY SINGLE PARASITES OF *PLASMODIUM GALLINACEUM*

Thirteen birds became infected out of forty inoculated with single erythrocytes containing single parasites. Downs (1947) obtained only three infections in forty-six birds similarly inoculated. Failure to produce an infection may have been due to a technical fault, such as the retention of the parasitized erythrocyte in the needle or syringe, or to the immune response of the bird. It is possible also that some of the parasites injected were potential gametocytes and thus non-infective, but, since gametocytes were rare in the infections from which the isolations were made, this is not a probable source of failure.

The incubation period, as determined by a daily 3 min. examination of blood films, was 7–13 days, but parasites were at first very rare. On the 16th day following inoculation 1·0 ml. of blood from each of the birds in which no parasites had been found was inoculated into two chicks, but no chick thus inoculated became infected. It can therefore be concluded, as previously recorded by Downs, that cryptic infections do not occur. In the three birds infected by Downs the incubation period varied from 9 to 14 days.

The initial infections produced by the inoculation of single parasites differed from those produced by the inoculation of a number of parasites in that there was complete synchronism of growth and multiplication, but this sharply defined periodicity was gradually lost. The fully developed infections were lighter than normal in only three birds, and seven birds died from the effects of heavy parasitaemia. The observations made by Downs, that gametocytes of both sexes occurred in infections arising from single parasites and that exoerythrocytic parasites were present in the primary infections, were confirmed.

IV. THE EFFECT OF A SINGLE COURSE OR OF TWO COURSES OF TREATMENT WITH METACHLORIDINE UPON *PLASMODIUM GALLINACEUM*

The effect of a single course of treatment with 0·05 mg./20 g. of metachloridine was studied in thirty groups containing a total of ninety-one birds, each bird being inoculated with 5×10^7 parasites* of clone A. 0·05 mg./20 g. was the lowest dose

* Throughout these experiments the numbers of parasites in the inocula are approximate.

studied, since it had been found previously that in birds treated with 0·025 mg./20 g. doses the intensity of infection on the 4th day was very variable (Bishop & McConnachie, 1953). In the majority of the birds treated with 0·05 mg./20 g. doses, a few parasites were found on the 4th day following inoculation, but the majority were abnormal in appearance. In the 500 erythrocyte samples examined from a few birds no parasites were found, but in four they were more numerous though obviously affected by the drug. Similar results were obtained in fifty-five birds inoculated with 10^8 parasites and treated with the same dose of drug, and also in ninety-one birds inoculated with 5×10^7 parasites and in seventy-one inoculated with 10^8 parasites and treated with 0·1 mg./20 g. doses of the drug, with the exception that the numbers of surviving parasites tended to be greater in birds treated with the lower dose of the drug and inoculated with the larger number of parasites. Out of a total of 308 birds receiving single courses of treatment with the drug, 249 (80 %) had less than twenty parasitized erythrocytes per 500 on the 4th day following inoculation, and only seven had more than forty, these latter being birds which had been inoculated with 10^8 parasites and treated with 0·05 mg./20 g.

In groups of birds inoculated intravenously with 5×10^7 parasites the parasite count in blood films made 15 min. after inoculation was only 1–2 p.e./500 e., whereas 4 days later, after a course of treatment with 0·1 mg./20 g. doses of the drug, it varied from 6 to 12 p.e./500 e. Some multiplication of the parasites must therefore have occurred during drug treatment.

In order to discover whether the survivors of a single course of treatment were more resistant to the drug than the normal population, the effect of a second course of treatment was studied in clone A. Blood from birds which had received one course of treatment was inoculated into untreated birds and, after the parasites had multiplied, groups of birds were inoculated from them and received a course of treatment. As in the previous experiments, inocula of 5×10^7 and 10^8 parasites were used, the courses of treatment consisting of 0·05 mg./20 g. doses in sixteen birds and 0·1 mg./20 g. in fifty-six birds. Of the total of seventy-two birds inoculated with parasites derived from birds which had undergone a course of treatment, fifty-six (77 %) had less than twenty parasitized erythrocytes per 500 on the 4th day following inoculation and only four had more than forty. Even when the parasites in the inocula were derived from birds which, at the end of the first treatment, had a comparatively large number of surviving parasites, no indication of an enhancement of resistance to the drug could be detected. The majority of the surviving parasites, as in experiments with a single course of drug treatment, were abnormal in appearance.

It was observed that the development of infections produced in untreated birds by parasites which had survived a single course of drug treatment was at first much slower than that of infections produced by similar numbers of normal parasites, but the rate of the increase in numbers of parasites gradually quickened as the infections progressed. In the early stages of such infections many of the parasites which were undergoing schizogonic division appeared to be abnormal, but later no abnormal forms were observed. It can therefore be concluded, both on

morphological grounds and from observations upon the rate of multiplication, that many of the parasites observed at the end of a single course of treatment with metachloridine were damaged by the drug but were capable of multiplying in its absence.

Similar experiments were performed with clone B. Of sixty-six birds inoculated with 5×10^7 or 10^8 parasites and treated with 0·05 mg./20 g. doses of metachloridine, 38 had infections of less than 20 p.e./500 e., and of thirty-four birds inoculated with similar numbers of parasites and treated with 0·1 mg./20 g. doses, twenty-eight also had infections of less than this number, as had nine out of thirteen birds inoculated with $2{\cdot}5 \times 10^8$ parasites and treated with the latter dose of drug. In a further series of experiments the effect of a second course of treatment with the drug was studied with this clone. In these experiments twenty-two out of twenty-seven birds inoculated with 5×10^7 or 10^8 parasites, derived from birds which had undergone a course of treatment with 0·05 mg./20 g. doses and treated with 0·05 mg./20 g. doses of the drug, had fewer than 20 p.e./500 e. at the end of the course of treatment, as had all ten birds inoculated with 5×10^7 parasites and treated with 0·1 mg./20 g. doses of the drug. A further test was made with twenty-six birds each inoculated with 5×10^7 parasites derived from birds which had been inoculated with $2{\cdot}5 \times 10^8$ parasites and treated with 0·1 mg./20 g. doses of the drug. After a course of treatment with 0·1 mg./20 g. doses of the drug, the second to which the strains had been exposed, all had infections of less than 20 p.e./500 e., twenty-five of them having fewer than ten parasitized erythrocytes per 500.

In these experiments, therefore, no difference was observed in the intensity of infection produced in birds undergoing a course of treatment with the drug, by parasites of the untreated parent clone and the progeny of parasites which had undergone a previous course of treatment. Resistance to metachloridine could not be detected after one course of treatment with the drug.

V. THE EFFECT OF SERIAL TREATMENT WITH 0·1 mg./20 g. DOSES OF METACHLORIDINE UPON THE DEVELOPMENT OF RESISTANCE TO THE DRUG IN CLONE A (STRAIN I)

Since resistant infections were not obtained amongst the 148 birds inoculated with strains which had been exposed to a previous course of treatment with the drug, a substrain of the untreated clone A was exposed to a series of courses of treatment with 0·1 mg./20 g. doses of metachloridine. A group of seven birds was inoculated with parasites of clone A; three received 5×10^7 parasites and served as controls, and four received 10^8 parasites and were treated with 0·1 mg./20 g. doses of metachloridine for $3\frac{1}{2}$ days (p. 212). The mean intensity of infection in the four treated birds on the 4th day following inoculation was 14 p.e./500 e. (Table 1) and, on this day, 1 ml. of heparinized blood from each of the birds was inoculated into clean birds, each bird receiving blood from a different donor. The first bird to develop a heavy infection was used as the donor for the next series of inoculations. Seven birds were inoculated and treated as in the previous experiment. No significant increase in the survival of the parasites was observed at the end of this second course of treatment, and, as before, heparinized blood from each bird was

inoculated into untreated birds. In the next experiment a group of eleven birds was inoculated; four of the birds received 10^8 parasites each and were treated with 0·1 mg. doses of the drug, and of the remaining seven birds, all of which were inoculated with 5×10^7 parasites, three served as untreated controls and four were treated with 0·1 mg. doses of the drug for comparison with the controls. In neither group of treated birds was any significant increase in resistance to the drug observed as the result of this third treatment. Blood from the birds inoculated with 10^8 parasites was inoculated into clean birds, one of which served as the donor for a fourth experiment; but again no increase in the numbers of surviving parasites was observed in the treated birds. In the fifth experiment two groups of eleven birds were inoculated, each with blood from a different untreated bird, and therefore with the progeny of the surviving parasites from two different treated birds. In each of these groups, both in the birds inoculated with 5×10^7 parasites and those inoculated with 10^8 parasites, a big increase in the numbers of surviving parasites was observed, though the mean number was considerably

Table 1. *The effect of serial treatments with* 0·1 *mg./*20 *g. metachloridine upon the development of resistance to the drug in* Plasmodium gallinaceum *clone A (strain I)*

Inoculum	Dose	No. of serial treatments 1	2	3	4	5	
		Mean no. of parasitized erythrocytes/500					
Drug-treated strain							
5×10^7	—	365	291	284	347	396	360
5×10^7	0·1	—	—	14	8	81	152
10^8	0·1	14	27	22	15	136	249
		Single treatments					
Untreated parent strain							
5×10^7	—	363	384	316	404	343	—
5×10^7	0·1	—	8	2	13	3	—
10^8	0·1	11	27	8	11	9	—

Inoculum in birds receiving serial courses of treatment with metachloridine = 10^8 parasites.

greater in one group than in the other (Table 1). In neither group, however, was the mean intensity of infection as great as in the untreated controls. In both groups the numbers were greater in the birds inoculated with 10^8 parasites than in those inoculated with 5×10^7, but in all the birds the parasites were normal in appearance. Although the intensity of infection varied considerably between the birds, from 55 to 233 p.e./500 e. in the birds inoculated with 5×10^7 parasites, and from 97 to 363 p.e./500 e. in those inoculated with 10^8 parasites, the lowest parasite counts were considerably higher than any observed previously.

Thus, in the fifth serial course of treatment with 0·1 mg./20 g. of metachloridine a sudden enhancement of resistance was apparent.

In each experiment, groups of birds inoculated with 5×10^7 and 10^8 parasites of the untreated parent strain, clone A, were given single courses of treatment with

0·1 mg./20 g. of metachloridine for comparison with the strain subjected to the serial treatments. The mean parasite counts on the 4th day varied from 3 to 13 p.e./500 e. in the birds inoculated with 5×10^7 parasites and 8–27 p.e./500 e. in those inoculated with 10^8 (Table 1).

In order to test whether the newly developed enhancement of resistance to the drug could be retained after mosquito transmission, a colony of *Aëdes aegypti* was fed upon one of the birds which showed a heavy infection with parasites, including gametocytes, at the end of the 5th serial treatment. These parasites were normal in appearance. The mosquitoes became heavily infected, and the sporozoites which developed produced infections which proved to have retained the enhanced resistance to metachloridine unimpaired.

VI. THE EFFECT OF SERIAL TREATMENT WITH 0·05 mg./20 g. DOSES OF METACHLORIDINE UPON THE DEVELOPMENT OF RESISTANCE TO THE DRUG IN CLONE A (STRAIN II)

A second series of experiments was carried out with the untreated clone A which differed from the former series in that the courses of treatment to which the parasites were exposed consisted of 0·05 mg./20 g. doses. As in the previous experiments inocula of 10^8 parasites were used for maintaining the drug-treated strain

Table 2. *The effect of serial treatments with* 0·05 *mg./*20 *g. metachloridine upon* Plasmodium gallinaceum *clone A* (*strain II*)

		No. of serial treatments						
Inoculum	Dose	1	2	3	4		5	6
		Mean no. of parasitized erythrocytes/500						
Drug-treated strain								
5×10^7	—	411	419	376	380	269	417	388
5×10^7	0·05	—	21	33	87	38	71	87
10^8	0·05	31	29	90	196	260	96	199
		Single treatments						
Untreated parent strain								
5×10^7	—	415	374	424	376	—	374	364
5×10^7	0·05	—	14	14	32	—	2	2
10^8	0·05	31	—	—	—	—	9	8

Inoculum in birds receiving serial courses of treatment with metachloridine = 10^8 parasites.

serially during drug treatment, but in each experiment birds were also inoculated with 5×10^7 parasites and treated with the same dose of drug for comparison with the untreated controls which received a similar inoculum (Table 2). A definite enhancement of resistance to the drug was observed at the end of the 3rd serial treatment in the group of birds inoculated with 10^8 parasites, though it was not apparent in those inoculated with 5×10^7. In the 4th serial treatment, two groups of eleven birds were inoculated with parasites derived from two different birds of the 3rd serial treatment. In both groups the birds inoculated with 10^8 parasites showed

a definite enhancement of resistance to the drug, but with those inoculated with 5×10^7 parasites the results were more variable though the parasites were normal in appearance. Even after the 6th serial treatment and in chicks inoculated with 10^8 parasites, the mean parasite count was not as great as in the untreated controls inoculated with 5×10^7 parasites. The variability of the intensity of infection amongst individual birds treated with this small dose of drug was, however, considerable, and once an enhancement of resistance to the drug had developed, some birds showed infections as heavy as those of the untreated controls; but when parasites from these heavily infected birds were inoculated into other birds and treated with the drug, the infections produced were not necessarily equally heavy.

Throughout the serial experiments groups of birds were inoculated with 5×10^7 or 10^8 parasites of the untreated parent clone A and given single courses of treatment with 0·05 mg./20 g. doses of the drug. The mean intensity of infection on the 4th day varied from 2 to 32 p.e./500 e. in the groups inoculated with 5×10^7 parasites and from 8 to 31 p.e./500 e. in those inoculated with 10^8 (Table 4).

VII. THE EFFECT OF SERIAL TREATMENT WITH 0·25 mg./20 g. DOSES OF METACHLORIDINE UPON THE DEVELOPMENT OF RESISTANCE TO THE DRUG IN CLONE A (STRAIN III)

The effect of a higher dose of metachloridine (0·25 mg./20 g.) upon the untreated parent strain was next studied. The form of the experiment was similar to that of the preceding ones. In each serial treatment, in addition to the group of birds inoculated with 10^8 parasites, a group was inoculated with 5×10^7 parasites and also treated with 0·25 mg./20 g. doses of the drug for comparison with the untreated controls which received similar inocula, and a second similar group was treated with 0·05 mg./20 g. doses, since it was considered possible that resistance to a lower dose might be more readily detected than resistance to the higher dose (Table 3). The parasites for the serial courses of treatment were, however, always taken from the groups of birds inoculated with 10^8 parasites and treated with 0·25 mg./20 g. doses of the drug (Table 3).

A sudden enhancement of resistance to the drug was observed after the 4th serial treatment. Although the heaviest infections occurred amongst the birds treated with 0·05 mg./20 g. doses, the infections were also heavy amongst the group inoculated with 10^8 parasites and treated with 0·25 mg./20 g. doses. The infections produced after the 5th serial treatment were lighter, but after the 6th and 7th treatments they increased again, one bird inoculated with 5×10^7 parasites and treated with 0·05 mg./20 g. having an infection of 406 p.e./500 e., which was heavier than the majority of infections produced in the untreated controls.

In each experiment groups of birds were inoculated with 5×10^7 or 10^8 parasites of the untreated parent strain and given a course of treatment with 0·05 or 0·25 mg./20 g. doses of the drug for comparison with the infections produced in the serial treatments. The maximum mean intensity of infection was 29 p.e./500 e. which occurred in the birds receiving the larger inoculum and the lower dose of drug (Table 3).

Table 3. *The effect of serial treatment with* 0·25 *mg./*20 *g. metachloridine upon* Plasmodium gallinaceum *clone A (strain III)*

Inoculum	Dose	No. of serial treatments 1	2	3	4	5	6	7
		Mean no. of parasitized erythrocytes/500						
Drug-treated strain								
5×10^7	—	375	403	377	370	401	385	402
5×10^7	0·05	—	3	10	223	94	198	267
5×10^7	0·25	1	—	2	71	51	81	64
10^8	0·25	3	6	5	193	29	174	—
		Single treatments						
Untreated parent strain								
5×10^7	—	392	178	382	419	422	—	—
5×10^7	0·05	20	3	4	4	3	—	—
5×10^7	0·25	1	2	2	2	2	—	—
10^8	0·05	29	8	—	5	—	—	—
10^8	0·25	5	2	—	5	—	—	—

Inoculum in birds receiving serial courses of treatment with metachloridine = 10^8 parasites.

VIII. A COMPARISON OF THE DEVELOPMENT OF RESISTANCE TO METACHLORIDINE IN STRAINS OF CLONE A TREATED WITH 0·05 mg./20 g. (STRAIN IV) AND 0·25 mg./20 g. DOSES OF METACHLORIDINE (STRAIN V) AND WITH 0·05 mg./20 g. (STRAIN VI) AND 1·0 mg./20 g. DOSES (STRAIN VII)

In order to discover whether the size of the dose of drug affected the rate of development of resistance to metachloridine, two strains of the untreated clone A, maintained under similar conditions, were respectively subjected to courses of treatment with 0·05 mg./20 g. doses (strain IV) and 0·25 mg./20 g. doses (strain V). In the serial passages through the drug-treated birds both strains were maintained by inocula of 10^8 parasites, but, as in the previous experiment, the effect of these doses of drug upon birds inoculated with 5×10^7 parasites was also studied. The time in which each strain was maintained in untreated birds, between the serial passages through birds receiving drug treatment, was identical for the two strains.

In strain IV no enhancement of resistance was evident until the end of the 4th serial treatment when an increase in the mean intensity of infection was observed in the birds inoculated with 10^8 parasites (Table 4), but the intensity of infection varied greatly in the four birds. At the end of the 5th serial treatment no significant enhancement of resistance was apparent, but after the 6th serial treatment very heavy infections, some of which were as heavy as those of the untreated controls, were observed in all the birds whether inoculated with 10^8 or 5×10^7 parasites.

In strain V a slight enhancement of resistance was apparent at the end of the 3rd serial treatment, which became more pronounced after a further treatment and very marked after the 5th serial treatment. In the 5th serial treatment, in addition to the birds inoculated with 5×10^8 and 5×10^7 parasites and treated with

Table 4. Plasmodium gallinaceum. *A comparison of the effect of serial treatments with 0·05 mg./20 g. doses and 0·25 mg./20 g. doses of metachloridine upon clone A (strains IV and V)*

		Strain IV No. of serial treatments						Strain V No. of serial treatments				
Inoculum	Dose	1	2	3	4	5	6	1	2	3	4	5
		Mean no. of parasitized erythrocytes/500										
Drug-treated strain												
5×10^7	—	178	429	356	373	292	433	178	388	360	400	413
5×10^7	0·05	3	2	26	20	26	343	—	—	—	—	438
10^8	0·05	8	15	31	103	38	388	—	—	—	—	—
5×10^7	0·25	—	—	—	—	—	—	2	6	59	94	282
10^8	0·25	—	—	—	—	—	—	2	4	70	256	363
		Single treatments										
Untreated parent strain												
5×10^7	—	383	334	374	419	407	—	—	—	—	—	—
5×10^7	0·05	4	5	15	5	10	—	—	—	—	—	—
5×10^7	0·25	2	—	3	2	—	—	—	—	—	—	—

Inoculum in birds receiving serial courses of treatment with metachloridine = 10^8 parasites.
Strain IV received serial courses of treatment with 0·05 mg./20 g. metachloridine.
Strain V received serial courses of treatment with 0·25 mg./20 g. metachloridine.

0·25 mg./20 g. doses of the drug, a group of birds was inoculated with 5×10^7 parasites per bird and treated with 0·05 mg./20 g. doses, and in all four birds the infections which developed were extremely heavy, the parasite count being greater than 400 p.e./500 e. and one as great as 470 p.e./500 e. In this series of experiments, therefore, resistance to the drug developed more rapidly and with greater regularity in the strain exposed to the serial courses of treatment with 0·25 mg./20 g. than in that exposed to treatment with 0·05 mg. doses.

As in the previous experiments groups of birds were inoculated with parasites of the untreated parent strain for comparison with the infections produced in the serial treatments. The highest mean parasite count, which occurred in a group inoculated with 5×10^7 parasites and treated with 0·05 mg./20 g. doses of the drug, was fifteen (Table 4).

In a further series of experiments two strains, each derived from the untreated parent strain, were treated, one (strain VI) with 0·05 mg./20 g. doses of the drug, and the other (strain VII) with 1·0 mg./20 g. doses. The serial inoculations into birds treated with these doses were made with 10^8 parasites, but since it was possible that, in the strain treated with 1·0 mg. doses, resistance to low doses might develop before resistance to 1·0 mg. doses, in each experiment a group of birds was inoculated with 5×10^7 parasites of this strain and treated with 0·05 mg./20 g. doses of the drug (Table 5).

An enhancement of resistance was first detected in strain VI, after the 4th serial treatment. The intensity of infection was high in all four birds inoculated with 10^8 parasites and treated with the drug. Although the infections were not so heavy in the group inoculated with 5×10^7 parasites, there was little variation between

individual birds. No enhancement of resistance could be detected at the end of the 4th serial treatment in strain VII, either in the birds inoculated with 10^8 parasites or in those inoculated with 5×10^7 parasites and treated with 1·0 mg./20 g. doses, or in those inoculated with 5×10^7 parasites and treated with 0·05 mg./20 g. doses. Unfortunately, it was impossible to continue the treatment of this strain for a longer period.

Table 5. Plasmodium gallinaceum. *A comparison of the effect of serial treatments with 0·05 mg./20 g. doses and 1·0 mg./20 g. doses of metachloridine upon clone A (strains VI and VII)*

		Strain VI No. of serial treatments				Strain VII No. of serial treatments			
Inoculum	Dose	1	2	3	4	1	2	3	4
		Mean no. of parasitized erythrocytes/500							
Drug-treated strain									
5×10^7	—	401	375	372	312	401	362	379	394
5×10^7	0·05	9	9	17	221	—	5	22	10
10^8	0·05	13	30	18	351	—	—	—	—
5×10^7	1·0	—	—	—	—	—	—	4	2
10^8	1·0	—	—	—	—	8	3	13	6
		Single treatments							
Untreated parent strain									
5×10^7	—	401	325	369	403	—	—	—	
5×10^7	0·05	9	10	16	15	—	—	—	—

Inoculum in birds receiving serial courses of treatment with metachloridine = 10^8 parasites.
Strain VI received serial courses of treatment with 0·05 mg./20 g. metachloridine.
Strain VII received serial courses of treatment with 1·0 mg./20 g. metachloridine.

In groups of birds inoculated with 5×10^7 parasites per bird of the untreated parent strain and subjected to a single course of treatment with 0·05 mg. doses of the drug, the highest mean count was 16 p.e./500 e. (Table 5).

IX. THE EFFECT OF VARIATIONS IN THE SIZE OF THE INOCULUM UPON THE RATE OF DEVELOPMENT OF RESISTANCE TO METACHLORIDINE IN CLONE A (STRAINS VIII, IX, X, XI AND XII)

Since the size of the dose of metachloridine did not appear to be a significant factor in determining the rate of development of resistance to the drug in infections induced by 10^8 parasites, the effect of inocula of 10^9 parasites, the largest number practicable, was studied. One strain (strain VIII), maintained by the inoculation of this number of parasites, was treated with 0·05 mg./20 g. doses of the drug, and a second similar strain (strain IX) was treated with 0·25 mg. doses. In each serial experiment a group of birds was inoculated with 5×10^7 parasites of strain VIII and treated with 0·05 mg. doses, and another group was inoculated with the same number of parasites of strain IX and also treated with 0·05 mg. doses of the drug. The sensitivity of the two strains to the same dose of drug could therefore be compared in infections produced by similar inocula (Table 6). In both strains an enhance-

ment of resistance was observed after the 3rd serial treatment, although the intensity of infection was greater and less variable in the group of birds inoculated with strain IX which had been subjected to serial courses of treatment with 0·25 mg./ 20 g. doses than in strain VIII which had been subjected to serial treatment with 0·05 mg./20 g. doses

Table 6. Plasmodium gallinaceum. *The effect of serial treatments with* 0·05 *mg. and* 0·25 *mg. doses of metachloridine upon clone A (strains VIII and IX) maintained by inocula of* 10^9 *parasites*

		Strain VIII No. of serial treatments			Strain IX No. of serial treatments		
Inoculum	Dose	1	2	3	1	2	3
		Mean no. of parasitized erythrocytes/500					
Drug-treated strain							
5×10^7	—	391	349	378	391	351	367
5×10^7	0·05	1	1	130	—	2	352
10^9	0·05	56	32	272	—	—	—
10^8	0·25	—	—	—	44	26	278
		Single treatments					
Untreated parent strain							
5×10^7	—	—	14	6	—	—	—

Strain VIII received serial courses of treatment with 0·05 mg./20 g. metachloridine.
Strain IX received serial courses of treatment with 0·25 mg./20 g. metachloridine.

In a further series of experiments the development of resistance to metachloridine was compared in three strains of clone A; strain X was maintained by the inoculation of 10^8 parasites in serial courses of treatment with 0·05 mg./20 g. of the drug; strain XI was maintained by inocula of a similar size in serial courses of treatment with 0·25 mg./20 g. doses, and strain XII was maintained by inocula of 10^9 parasites in serial courses of treatment with 0·25 mg./20 g. doses of the drug. In all three strains, in each experiment, in addition to the groups of birds undergoing serial courses of treatment and their controls, other groups, one for each strain, were inoculated with 5×10^7 parasites and treated with 0·05 mg./20 g. doses of the drug in order that the degree of resistance attained by each strain could be compared under similar conditions (Table 7). The period of time for growth in untreated birds was similar in all three strains. In strain X an increase in the mean intensity of infection was observed after the 4th serial course of treatment with 0·05 mg./20 g. doses. In strain XI an increase in the mean intensity of infection was observed after the 3rd serial course of treatment in the group of birds inoculated with 5×10^7 parasites and treated with 0·05 mg./20 g. doses, but in the group inoculated with 10^8 parasites and treated with 0·25 mg./20 g. doses there was little increase in the mean intensity of infection, though in one of the birds the parasite count was 91 p.e./500 e. After the 4th serial treatment, however, an enhancement of resistance was observed in both groups of birds. In strain XII a significant enhancement of resistance was first observed after the 4th serial course of treatment with 0·25 mg./20 g. doses of the drug. This strain was im-

mediately inoculated into birds treated with 1·0 mg./20 g. doses, and although the inocula consisted of only 5×10^7 parasites, infections varying from 182 to 358 p.e./500 e. were produced. The strain had thus become resistant to doses of metachloridine at least four times greater than that with which it had been treated. Higher doses were not tested.

Table 7. *The effect of serial treatments with* 0·05 *mg./20 g. doses of metachloridine upon a strain of* Plasmodium gallinaceum *(clone A) maintained by the inoculation of* 10^8 *parasites (strain X) and of* 0·25 *mg./20 g. doses upon strains maintained by the inoculation of* 10^8 *(strain XI) and* 10^9 *parasites (strain XII)*

		Strain X No. of serial treatments					Strain XI No. of serial treatments				Strain XII No. of serial treatments				
Inoculum	Dose	1	2	3	4	5	1	2	3	4	1	2	3	4	5
		Mean no. of parasitized erythrocytes/500													
Drug-treated strain															
5×10^7	—	414	324	376	321	388	414	347	404	382	414	372	412	388	351
5×10^7	0·05	—	2	3	141	196	—	14	131	344	—	8	12	344	—
10^8	0·05	10	15	4	194	—	—	—	—	—	—	—	—	—	—
5×10^7	0·25	—	—	—	—	—	—	—	—	—	—	—	—	—	187
10^8	0·25	—	—	—	—	—	6	10	32	146	—	—	—	—	—
10^9	0·25	—	—	—	—	—	—	—	—	—	64	38	73	249	—
5×10^7	1·0	—	—	—	—	—	—	—	—	—	—	—	—	—	273
		Single treatments													
Untreated parent strain															
5×10^7	—	248	391	413	415	401	—	—	—	—	—	—	—	—	—
5×10^7	0·05	1	2	12	14	18	—	—	—	—	—	—	—	—	—
10^8	0·05	—	—	—	—	21	—	—	—	—	—	—	—	—	—
10^9	0·05	—	56	—	—	—	—	—	—	—	—	—	—	—	—
10^9	0·25	—	44	—	—	—	—	—	—	—	—	—	—	—	—

The effect of single courses of treatment with 0·05 and 0·25 mg./20 g. was tested upon birds inoculated with 5×10^7, 10^8 and 10^9 parasites of the untreated parent clone A for comparison with the serial treatments. The intensity of the infections produced was within the normal range (Table 7).

X. THE EFFECT OF SERIAL COURSES OF TREATMENT WITH 0·1 mg./20 g. OF METACHLORIDINE UPON *PLASMODIUM GALLINACEUM* CLONE B (STRAIN XIII)

In a series of experiments with clone B the groups of birds receiving serial courses of treatment with 0·1 mg./20 g. doses of metachloridine were inoculated with 10^8 parasites, but from the third experiment onwards a group of birds which received similar doses of drug was inoculated with 5×10^7 parasites per bird, an inoculum similar to that of the untreated controls (Table 8). Although at the end of the second course of treatment the mean intensity of infection was higher than usual, no enhancement of resistance was observed at the end of the third treatment although the birds in this experiment were inoculated with parasites derived from a bird in the second experiment which had a count of 49 p.e./500 e. In the fifth

experiment two series of birds were treated in parallel, each being inoculated with parasites derived from a different donor bird from the group which had undergone the 4th serial course of treatment, and which had shown no enhancement of resistance to the drug. In the treated birds in both series the intensity of infection was high, one bird inoculated with 10^8 parasites having a parasite count of 408 p.e./500 e. The mean intensity of infection was, however, higher in one series than in

Table 8. Plasmodium gallinaceum. *The effect of serial treatments with* 0·1 *mg./20 g. doses of metachloridine upon the development of resistance to the drug in clone B (strain XIII)*

Inoculum	Dose	No. of serial treatments: 1	2	3	4	5		6
		Mean no. of parasitized erythrocytes/500						
Drug-treated strain								
5×10^7	—	375	397	348	339	319	341	270
5×10^7	0·1	—	—	13	7	328	183	268
5×10^7	0·2	—	—	—	—	—	—	262
10^8	0·1	5	45	10	11	395	254	—
		Single treatments						
Untreated parent strain								
5×10^7	—	363	384	316	404	343	—	320
5×10^7	0·1	—	8	2	13	3	—	11
10^8	0·1	11	27	8	11	9	—	—

Inoculum in birds receiving serial courses of treatment with metachloridine = 10^8 parasites.

the other. Parasites from the bird infected with 408 p.e./500 e. were inoculated into two groups of birds, each bird receiving 5×10^7 parasites; one group was treated with 0·1 mg./20 g. doses of the drug and the other with 0·2 mg./20 g. The infections produced in the group of birds treated with 0·2 mg./20 g., a dose twice as big as that with which the strain had previously been in contact, were as heavy as in the group treated with the lower dose.

Groups of birds were inoculated with 5×10^7 and 10^8 parasites of the untreated parent strain clone B, and given single courses of treatment with 0·1 mg./20 g. for comparison with the strain subjected to serial treatments. The intensities of infection were within the normal range (Table 8).

XI. THE EFFECT OF THE SIZE OF THE INOCULUM UPON THE RATE OF DEVELOPMENT OF RESISTANCE TO METACHLORIDINE IN *PLASMODIUM GALLINACEUM* CLONE B (STRAINS XIV AND XV)

A comparison was made of the rate of development of resistance to metachloridine in two strains of clone B treated with 0·1 mg./20 g. doses of the drug, one strain (strain XIV) being maintained through the serial courses of treatment by the inoculation of $2{\cdot}5 \times 10^7$ parasites and the other (strain XV) by the inoculation of $2{\cdot}5 \times 10^8$ parasites. The length of time passed in untreated birds was similar for both strains. In order that the rate of development of resistance could be compared under similar conditions, in each experiment, with both strains, groups of

birds were inoculated with 5×10^7 parasites and treated with 0·1 mg./20 g. doses (Table 9).

Strain XIV was subjected to seven serial courses of treatment with the drug, but although the parasite counts were slightly higher at the end than at the beginning, no definite enhancement of resistance was observed. In strain XV no enhancement of resistance was detected after the first two serial courses of treatment. In the next serial experiment, in addition to the group of birds inoculated with $2{\cdot}5 \times 10^8$ parasites, five groups of birds were inoculated with 5×10^7 parasites derived from different treated birds of the previous experiment. Three birds from each group served as controls, the remaining birds being treated with 0·1 mg./20 g. doses of the drug (Table 9). In two of these latter groups, and in the group inoculated with $2{\cdot}5 \times 10^8$ parasites, a definite enhancement of resistance was observed.

Table 9. Plasmodium gallinaceum. *Clone B. A comparison of the effect of serial courses of treatment with* 0·1 *mg./*20 *g. doses of metachloridine upon groups of birds inoculated with* $2{\cdot}5 \times 10^7$ *parasites* (*strain XIV*) *or* $2{\cdot}5 \times 10^8$ *parasites* (*strain XV*) *per bird*

		Strain XIV, no. of serial treatments							Strain XV, no. of serial treatments							
Inoculum	Dose	1	2	3	4	5	6	7	1	2	3					4
		Mean no. of parasitized erythrocytes/500														
Drug-treated strain																
5×10^7	—	254	394	353	395	352	411	388	303	337	438	326	378	377	286	294
$2{\cdot}5 \times 10^7$	0·1	1	2	1	6	6	16	—	—	—	—	—	—	—	—	—
5×10^7	0·1	3	8	3	12	17	38	23	—	3	75	60	35	33	3	105
$2{\cdot}5 \times 10^8$	0·1	—	—	—	—	—	—	—	19	22	—	—	—	129	—	311
		Single treatments														
Untreated parent strain																
5×10^7	—	281	300	346	182	424	352	355	323	—	—	—	—	—	—	—
5×10^7	0·1	2	10	10	2	6	2	4	17	—	—	—	—	—	—	—

In two other groups, although the mean intensity of infection was only 33 and 35 p.e./500 e., one of the birds had a parasite count of 75 p.e./500 e. and two others had counts of 50 and 53. In the fifth group, however, the parasite counts were very low. Parasites derived from one of the birds in the group inoculated with $2{\cdot}5 \times 10^8$ parasites were inoculated into two groups of birds, one group receiving $2{\cdot}5 \times 10^8$ parasites and the other 5×10^7, and subjected to a further course of treatment after which resistance was found to have increased.

Throughout the series of experiments the effect of single treatments with 0·1 mg./20 g. doses of the drug were studied for comparison with the serial treatments. The parasite counts on the fourth day were low (Table 9).

XII. THE EFFECT OF INOCULA CONSISTING OF MIXTURES OF METACHLORIDINE-RESISTANT AND NORMAL PARASITES

In the foregoing experiments, strains of two clones of *P. gallinaceum*, maintained by inocula varying from $2{\cdot}5 \times 10^7$ to 10^9 parasites, were subjected to courses of treatment with metachloridine, but in no case was a definite enhancement of

resistance, that is to say, a significant increase in number of parasites normal in appearance, observed before the end of the 3rd serial course of treatment. In order to discover how long it took for resistant parasites to overgrow a normal population, and also the smallest number of resistant parasites which could be selected from such a population, a series of experiments was carried out in which the inocula consisted of a mixture of a strain of clone A resistant to 1·0 mg./20 g. metachloridine and the normal parent strain. In these experiments blood infected with the metachloridine-resistant strain was diluted with citrate-saline solution so that 0·1 ml. contained approximately $1–10^6$ parasites. Although birds with light infections, in which the majority of parasitized erythrocytes contained only one parasite, were selected as donors, some of the erythrocytes doubtless contained more than one parasite but this error in dilution was unavoidable. Blood infected with the untreated parent strain was diluted so that 0·25 ml. contained 5×10^7 parasites. Each bird was inoculated intravenously with this number of parasites of the parent strain and with $1–10^6$ parasites of the metachloridine-resistant strain. The birds, with the exception of three untreated controls, were given a course of treatment with 0·1 mg./20 g. doses of the drug, the first dose being given immediately after inoculation. In each experiment, in addition to the birds inoculated with the mixture of parasites of the parent and metachloridine-resistant strains, a group of seven birds was inoculated with 5×10^7 parasites of the parent strain per bird, three of the birds serving as untreated controls and the remainder being treated with 0·1 mg./20 g. doses of the drug, and another group of seven birds was inoculated with the metachloridine-resistant strain and similarly treated (Table 10).

Table 10

	1st treatment				2nd treatment			
	Inoculum		Dose		Inoculum		Dose	
Expt.	P	Met R	0	0·1	P	P+Met R	0	0·1
1	5×10^7	—	329*	5*	5×10^7	—	344* 418	14* 4
2	5×10^7	—	361	2	5×10^7	—	376	7
3	5×10^7	—	354	4	5×10^7	—	382	6
4	5×10^7	—	309	8	5×10^7	—	349 331	12 8
5	5×10^7	—	382	12	5×10^7	—	362 433 314	5 4 2
1	—	5×10^7	385	406	—	—	—	—
2	—	5×10^7	412	426	—	—	—	—
3	—	5×10^7	340	343	—	—	—	—
4	—	5×10^7	392	393	—	—	—	—
5	—	5×10^7	389	356	—	—	—	—
1	5×10^7	10^6	—	18	—	—	—	—
2	5×10^7	10^6	—	39	—	—	—	—
3	5×10^7	10^6	—	21	—	—	—	—
1	5×10^7	10^5	—	7	—	—	—	—
2	5×10^7	10^5	—	10	—	—	—	—
3	5×10^7	10^5	—	2	—	—	—	—

Table 10 (*cont.*)

	1st treatment				2nd treatment			
	Inoculum		Dose		Inoculum		Dose	
Expt.	P	Met R	0	0·1	P	P+Met R	0	0·1
1	5×10^7	10^4	—	15	—	5×10^7	372 364	334 161
2	5×10^7	10^4	—	4	—	—	—	—
3	5×10^7	10^4	—	10	—	—	—	—
1	5×10^7	10^3	—	15	—	5×10^7	323 359 275	22 182 9
2	5×10^7	10^3	—	1	—	5×10^7	370 415 428	330 438 437
3	5×10^7	10^3	—	6	—	5×10^7	331	329
4	5×10^7	10^3	—	7	—	5×10^7	392	206
5	5×10^7	10^3	—	6	—	5×10^7	368	364
1	5×10^7	10^2	—	10	—	5×10^7	369 398	24 19
3	5×10^7	10^2	—	6	—	5×10^7	381 311 323	59 198 184
4	5×10^7	10^2	—	5	—	5×10^7	355	51
4	5×10^7	10	—	7	—	5×10^7	301	2
5	5×10^7	10	—	8	—	5×10^7	373 395	25 39
4	5×10^7	1	—	10	—	5×10^7	409 299 374	13 2 21
5	5×10^7	1	—	8	—	—	397 238 427 379 325 283	245 8 160 166 61 18

* Mean number of parasitized erythrocytes/500 erythrocytes in groups of three to six birds.
Dose = metachloridine in mg./20 g. body weight of bird.
P = untreated parent strain clone A.
Met R = metachloridine-resistant strain clone A.
P + Met R = parasites derived from birds inoculated with a mixture of 5×10^7 parasites of the untreated parent strain and a known number of metachloridine-resistant parasites and treated with 0·1 mg./20 g. doses of the drug.

In three experiments groups of birds inoculated with a mixture of 5×10^7 parasites of the parent strain and 10^6 parasites of the metachloridine-resistant strain showed slightly higher parasite counts at the end of a course of treatment with the drug than those inoculated with the untreated parent clone A alone and receiving similar drug treatment, but not higher than had occasionally been seen with this strain under similar treatment; but the majority of the surviving parasites were normal in appearance. When the inoculum contained a smaller number of

metachloridine-resistant parasites, however, the parasite counts on the 4th day following inoculation did not differ significantly from those of the untreated parent strain in birds receiving similar doses of the drug.

In experiments in which birds were inoculated with 10^4 or fewer parasites of the metachloridine-resistant strain, blood from birds which had undergone a course of treatment with 0·1 mg./20 g. doses of the drug was inoculated on the 4th day, into untreated birds, each bird receiving 1 ml. of blood from a different donor. When sufficiently heavy infections had developed, blood from each bird was inoculated into a different group of birds, each bird receiving 5×10^7 parasites, and a course of treatment with 0·1 mg./20 g. doses of metachloridine, the second to which the parasites had been exposed, was carried out. Resistant infections were observed in two groups of birds inoculated with parasites derived from birds inoculated with a mixture containing 10^4 metachloridine-resistant parasites, though the mean parasite count was twice as high in one group as in the other (Table 10). In five similar experiments in which the number of metachloridine-resistant parasites inoculated per bird in the first course of treatment was 10^3, seven out of nine groups of birds, each inoculated with parasites derived from a different bird which had undergone the first course of treatment, showed resistant infections. With inocula of 10^2 metachloridine-resistant parasites, though the parasite counts at the end of the second course of treatment were sufficiently high in four out of six groups of birds to show that resistant parasites were present, they were lower and more variable than in the previous experiment. With inocula of ten metachloridine-resistant parasites two out of three groups of birds had mean parasite counts at the end of the second course of treatment which were higher than those of the untreated parent strain, but they were not higher than had occasionally been observed with this strain after similar treatment. However, in four out of nine groups of birds, each inoculated with parasites derived from a different bird which had been inoculated with a single parasitized erythrocyte as assessed by dilution, resistant infections, though of varying intensity, were produced (Table 10).

XIII. DISCUSSION

In assessing and attempting to explain the data obtained from the serial treatment of strains of *P. gallinaceum* with metachloridine certain salient points emerge. First, resistant infections were never obtained as a result of one or two courses of treatment with the drug; they were, however, obtained after three to five courses of treatment in thirteen out of fifteen strains. Of the two strains in which no resistance was observed, one, treated with the highest dose of drug (1·0 mg./20 g.), had to be abandoned after the 4th serial treatment. Although only three strains of clone B were studied as compared with twelve of clone A, the results indicate that resistance to metachloridine developed with equal facility and in a similar manner in both strains. The number of courses of treatment required to produce resistant parasites could not be strictly related to the number of parasites in the inoculum, though in strains maintained with inocula containing the largest number (10^9), an enhancement of resistance was observed after the minimum

number of treatments (three), whereas the only failure was in a strain maintained by inocula containing the smallest number of parasites ($2{\cdot}5 \times 10^7$). The number of courses of treatment required to produce a resistant strain did not appear to be related to the size of the dose of drug, which varied from 0·05 mg./20 g. to 1·0 mg./20 g. In one strain treated with 0·25 mg./20 g. doses of the drug resistance to 0·05 mg./20 g. was detected before resistance to the higher dose, but in others the parasites became resistant to the higher dose without any intermediate steps being observed. Two strains were found to be resistant to doses greater than those to which they had been exposed, in one case to at least twice the dose and in the other to at least four times the dose. Complete uniformity in the pattern of development of resistance to the drug was not observed; in some strains a sudden big increase in the intensity of infections produced in treated birds was observed, whereas in others resistance developed more gradually.

There are three principal explanations of the methods by which drug resistance may arise in micro-organisms: (1) spontaneous mutation followed by selection of the drug-resistant mutants by the drug, (2) drug-induced mutation followed by selection, or (3) phenotypic adaptation. Whereas spontaneous mutations are relatively rare events—one mutant in 10^8 cells is the figure given for some bacteria—in phenotypic adaptation the change may occur in a large proportion of the population exposed to the drug.

If resistance to metachloridine in *P. gallinaceum* arose by spontaneous mutation, three possibilities exist: the mutants may have been present in the normal population, they may have arisen during the periods of uninhibited multiplication between courses of treatment, or they may have arisen during the actual course of treatment. Metachloridine is slow in acting, and since parasite counts were slightly higher at the end of a course of treatment with 0·1 mg./20 g. doses of the drug than at the beginning, some multiplication of the parasites must have taken place; mutation during drug treatment would therefore have been possible.

In experiments with inocula containing mixtures of known numbers of metachloridine-resistant and normal parasites, it was observed that an inoculum containing fewer than 10^6 resistant parasites failed to produce an increase in the parasite count as compared with the normal strain alone, at the end of a course of treatment with 0·1 mg./20 g. doses of the drug. It is, therefore, obvious that if resistant parasites occurred spontaneously in populations of normal parasites, an enhancement of resistance would not be detected at the end of a single course of treatment with the drug in infections of the normal parent strain produced by inocula of the size used in these experiments unless the proportion of resistant parasites was high. Since, however, the introduction of a single parasitized erythrocyte, as assessed by dilution, containing one, or possibly more, metachloridine-resistant parasites in an inoculum of 5×10^7 parasites of the normal parent strain can produce a significant increase in the parasite count at the end of the second course of treatment, and since, in the serial courses of treatment, an enhancement of resistance was never observed before the end of the third course, resistant parasites, if present in the normal population, must have been fewer than 1 in 50 million. Indeed, the observation that in three serial experiments inocula of

10^9 normal parasites did not produce an increase in the parasite count until the end of the third course of treatment, suggests that resistant parasites, if present in the normal population, may be fewer than 1 in 10^9.

The length of the asexual cycle of *P. gallinaceum* was found by Giovannola (1938) to be 36 hr., an observation which has been confirmed by Lumsden & Bertram (1940) and Taliaferro & Taliaferro (1950). The number of merozoites produced by schizonts in initial infections in chickens was found by the latter workers to range from four to forty, the merozoite mean throughout infection varying from fourteen to twenty-six, though during the acute rise it was usually high. Since a course of treatment with metachloridine covered a period of approximately 90 hr., two complete cycles of development could take place. Assuming that the number of merozoites produced at each schizogony was twenty-six, the total number of parasites which theoretically could be produced from one parasite during a course of treatment, if totally unaffected by the drug, would be 676, but if the number of merozoites was forty the total number would be 1600. The effect of innate immunity upon the survival of the parasites is, however, not known. Since the total number of parasites produced would be distributed throughout the total volume of blood, it is obvious that they would not significantly affect a parasite count based on 500 erythrocytes.

Since some sensitive parasites survive a course of treatment, the population at the end of a course of treatment, in a bird which had been inoculated with one or more resistant parasites and a large number of sensitive parasites, would consist of the progeny of the resistant parasite and the sensitive survivors. Both the resistant and sensitive parasites would grow in untreated birds, their final proportions depending upon their relative growth rates as well as upon the relative numbers introduced into the bird. The gradual development of resistant infections, as in strain V, Table 4, could be explained by an increasing proportion of resistant to sensitive parasites in consecutive inocula.

In some of the serial experiments a marked enhancement of resistance appeared suddenly. In these experiments the parasite counts of all the birds at the end of the previous course of treatment were low and gave no indication of a change in sensitivity to the drug. The fact, however, that after these parasites had multiplied in untreated birds they gave rise to heavy infections in treated birds suggested that resistant parasites were present. Moreover, in two experiments, the sudden appearance of resistant infections in two groups of birds inoculated with parasites derived from different birds subjected to the previous course of treatment gave support to this supposition (Tables 1 and 8). Further evidence was provided by the observation that the parasites at the end of courses of treatment prior to those in which the parasite count rose suddenly, showed little if any damage from the action of the drug, and upon inoculation into untreated birds produced heavy infections much more rapidly than was usual with the sensitive survivors of a course of treatment. It can, therefore, be concluded that resistant parasites were present at the end of the course of treatment prior to that in which the sudden enhancement was observed, but were too few to affect the parasite count. In the experiments with mixed infections, an inoculum of 10^3 parasites was necessary in the first course of

treatment, in order to produce a heavy infection at the end of the second course of treatment. These results, therefore, suggest that in the serial experiments in which an enhancement of resistance appeared suddenly, the resistant parasites originated at an earlier stage than in the previous course of treatment.

In an experiment (Table 9) in which five groups of birds were inoculated, each with parasites derived from a different bird from the group previously treated with the drug, the mean parasite counts showed a definite enhancement of resistance in two groups and a less marked enhancement in two others, whereas in the fifth group the count was very low. These results suggest that resistant parasites were present in four of the donors, but were absent or very few in the fifth. It suggests that resistance arises sporadically.

It was several times observed that when an enhancement of resistance appeared, the parasite counts amongst donor birds within a group were more variable than after the strain had undergone further treatment with the drug. It has been pointed out by Curd, Davey & Rose (1945) that at a border-line dose of an anti-malarial drug the variation in the parasite counts in different birds is very great. It is possible that, when resistance to metachloridine first arose in some strains it was very narrowly adjusted to a certain maximum concentration of the drug in the blood, and that variations in the peak concentration in different birds had a considerable effect upon the growth of the parasites. Such variations may have been the cause of a temporary decrease in parasite numbers in some strains (strains III and IV) after resistance had appeared to have been established.

That resistance to metachloridine, once it can be detected, may be a very stable character was shown in a strain treated with 0·1 mg./20 g. doses of the drug. The newly resistant strain retained its resistance to the drug undiminished after passage through untreated birds for 43 days, and after transmission through mosquitoes which involved a sexual cycle of development in the parasites. A passage through the insect vector entails profound changes in the environment of the parasite involving growth at a lower temperature and in cells of an entirely different type.

Although no definite proof has been obtained that resistance to metachloridine is produced by mutation of the parasite, the data obtained from serial treatments does not conflict with such a hypothesis. Indeed, the method of producing resistance by exposing the parasites to short periods of drug treatment alternating with longer periods of growth in untreated birds, is not suited to the development of an adaptation. Thus, in bacteria a characteristic of recently adapted cells is that they readily lose the adaptation on subculture on a drug-free medium (Dean & Hinshelwood, 1957). It would, for instance, be difficult to explain the sudden increase in resistance, seen in strain IX and in a substrain of strain XIII by adaptation. When resistance was first detected in these strains the mean parasite counts in birds inoculated with the same number of parasites was as high in treated as in untreated birds. The parasites in the inocula must therefore have been fully adapted to the concentration of the drug to which they were exposed. Since a period of growth in untreated birds immediately preceded the appearance of resistance, adaptation, if it occurred, must be assumed to have taken place in the

previous course of treatment. If this were so, it was not sufficiently developed to affect the parasite counts in these birds, yet, after a period of growth in untreated birds, the adapted parasites were able to produce heavy infections when inoculated into birds treated with the drug.

It is difficult to compare the rate of the development of resistance to metachloridine in *P. gallinaceum* in these experiments with the rate with which resistance to other drugs has been produced in this species since the methods of producing it have differed. A similar technique was, however, used by Williamson & Lourie (1947) in producing resistance to proguanil (*N'*-*p*-chlorophenyl-N^5-*iso*propyl-biguanide) in a mixed strain with the exception that, at the end of each course of treatment, the parasites were allowed to multiply in the birds in which they had been exposed to the drug before serving as donors to other birds undergoing drug treatment. Since resistance was first observed in the 13th week of the experiment after eight courses of treatment in one strain and in the 14th week after five courses of treatment with a larger dose in another, it is obvious that resistance to proguanil developed more slowly than resistance to metachloridine. In both strains, however, the increase in resistance occurred suddenly as in several of the metachloridine-resistant strains. It thus suggests a mutation with a much lower frequency than the experiments with metachloridine suggest.

Recently, the sudden appearance of an enhanced resistance to pyrimethamine (5-(*p*-chlorophenyl)-2:4-diamino-6-ethyl-pyrimidine) was observed in a patient who was receiving malaria therapy for neurosyphilis. The strain of *P. malariae* responded well to the drug in fourteen patients treated previously, but thereafter, in six others, showed varying degrees of resistance, usually of a high order (Young, 1957). In this strain also the abrupt appearance of an increased resistance to the drug suggests a mutation.

Of the drugs other than metachloridine which have been tested for the production of resistance in *P. gallinaceum*, only sulphadiazine had a relatively rapid effect. This drug produced a high degree of resistance to proguanil, though not to sulphadiazine itself, in one strain after 17 days' treatment, but the conditions of the experiment were not such as to show if resistance appeared suddenly.

Great difficulty was experienced in obtaining resistance to pamaquin and chloroquine in *P. gallinaceum*. The first slight enhancement of resistance to pamaquin was observed after 5 months' treatment with the drug (Bishop & McConnachie, 1952*b*). In one series of experiments with chloroquine no enhancement of resistance to this drug could be detected after 16 months' treatment (Bishop & McConnachie, 1952*a*), and in another a twofold enhancement was obtained after fifty-seven passages over a period of 9 months (Ray, Sharma & Misra, 1956). Although the conditions of the experiments were different from those with metachloridine, the development of resistance to these drugs was so slow that it must be concluded that if it arises by mutation, mutants must occur very rarely. It is obvious that the rate of production of resistance to metachloridine is extremely high as compared with that of other anti-malarial compounds which have been studied, but as yet the data are too limited to indicate whether it has a mutagenic action.

XIV. SUMMARY

1. The development of resistance to metachloridine (3-metanilamido-5-chloropyrimidine) was studied in two clones of *Plasmodium gallinaceum* derived from single erythrocytic parasites and maintained by serial inoculation in young chicks. Resistance developed with equal facility and similarly in the two clones.

2. In thirteen strains of these clones an enhancement of resistance was obtained after three to five courses of treatment, each of seven doses of metachloridine over a period of $3\frac{1}{2}$ days.

3. The effect of the size of the inoculum ($2{\cdot}5 \times 10^7$–10^9 parasites), and of the dose of drug upon the rate of development of resistance was studied. Although the number of courses of treatment required to produce an enhancement of resistance was not always related to the size of the inoculum, with the largest inoculum an enhancement of resistance was observed after the minimum number of courses of treatment (three), whereas with the smallest inoculum no enhancement of resistance was obtained. The rate of the development of resistance did not appear to be related to the size of the dose of drug.

4. In some strains the increase in resistance was sudden, whereas in others it was more gradual. Resistance was retained when parasites of a newly resistant strain were transmitted through *Aëdes aegypti* or maintained for 43 days in the absence of the drug.

5. A comparison of the development of resistance in populations of normal parasites, and of populations composed of mixtures of known numbers of resistant and normal parasites, indicated that the pattern of the development of resistance in normal populations could be explained by the selections of mutations of a frequency of less than 1 in 5×10^7, or probably less than 1 in 10^9 parasites.

REFERENCES

Bishop, A. & McConnachie, E. W. (1950). Sulphadiazine-resistance in *Plasmodium gallinaceum* and its relation to other antimalarial compounds. *Parasitology*, **40**, 163.

Bishop, A. & McConnachie, E. W. (1952*a*). Failure to produce resistance to chloroquine in *Plasmodium gallinaceum* in chicks. *Parasitology*, **42**, 52.

Bishop, A. & McConnachie, E. W. (1952*b*). Pamaquin resistance in a strain of *Plasmodium gallinaceum* and its relationship to other antimalarial drugs. *Parasitology*, **42**, 57.

Bishop, A. & McConnachie, E. W. (1953). The development of resistance to metachloride in *Plasmodium gallinaceum* in chicks. *Parasitology*, **42**, 277.

Coulston, F. & Manwell, R. D. (1941). Single-parasite infections and exoerythrocytic schizogony in *Plasmodium circumflexum*. *Amer. J. Hyg.* **34**, 119.

Curd, F. H. S., Davey, D. G. & Rose, F. L. (1945). Studies on synthetic antimalarial drugs. I. Biological methods. *Ann. Trop. Med. Parasit.* **39**, 139.

Dean, A. C. R. & Hinshelwood, Sir Cyril (1957). Aspects of the problem of drug resistance in bacteria. Ciba Foundation symposium on *Drug resistance in Microorganisms*. London: J. & A. Churchill Ltd.

Demidowa, L. W. (1934). Ueber die geringste zur Erzeugung der experimentellen Malaria nötige *Plasmodium gallinaceum*. *G. Batt. Immun.* **13**, 872.

Downs, W. G. (1947). Infections of chicks with single parasites of *Plasmodium gallinaceum* Brumpt. *Amer. J. Hyg.* **46**, 41.

Giovannola, A. (1938). Il *Plasmodium gallinaceum* Brumpt 1935, i così detti corpi toxoplasma-simili ed alcune inclusioni di probabile natura parassitaria nei globuli bianchi del *Gallus gallus*. *Riv. Parassit.* **2**, 129.

LUMSDEN, W. H. R. & BERTRAM, D. S. (1940). Observations on the biology of *Plasmodium gallinaceum* Brumpt, 1935, in the domestic fowl, with special reference to the production of gametocytes and their development in *Aëdes aegypti* (L). *Ann. Trop. Med. Parasit.* **34**, 135.

RAY, A. P., SHARMA, G. K. & MISRA, B. G. (1956). Screening of antimalarials against *P. gallinaceum* in chicks. VI. Evidence of two-fold acquired resistance to chloroquine diphosphate in a strain of *P. gallinaceum* in chicks. *Ind. J. Malar.* **10**, 299.

SONNEBORN, T. M. (1947). Recent advances in the genetics of Paramecium and Euplotes. *Advanc. Genet.* **1**, 263.

TALIAFERRO, W. H. & TALIAFERRO, L. G. (1950). Reproduction-inhibiting and parasiticidal effects of *Plasmodium gallinaceum* and *Plasmodium lophurae* during initial infection and homologous superinfection in chickens. *J. Infect. Dis.* **86**, 275.

WILLIAMSON, J. & LOURIE, E. M. (1947). Acquired paludrine-resistance in *Plasmodium gallinaceum*. I. Development of resistance to paludrine and failure to develop resistance to certain other antimalarials. *Ann. trop. Med. Parasit.* **41**, 278.

YOUNG, M. D. (1957). Resistance of *Plasmodium malariae* to pyrimethamince (daraprim). *Amer. J. Trop. Med.* **6**, 621.

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